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Genetic Diversity Analysis of 11 Black Walnut (Juglans Nigra L.) Cultivars and Development of a Mapping Population

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GENETIC DIVERSITY ANALYSIS OF 11 BLACK WALNUT (*JUGLANS NIGRA* L.) CULTIVARS AND DEVELOPMENT OF A MAPPING POPULATION

A Master's Thesis

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Master of Science, Plant Science

By

Steven J. Schneider, Jr

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GENETIC DIVERSITY ANALYSIS OF 11 BLACK WALNUT (*JUGLANS NIGRA* L.) CULTIVARS AND DEVELOPMENT OF A MAPPING POPULATION

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ABSTRACT

Eastern black walnut (*Juglans nigra* L.) production stands on the brink of potentially great market expansion that can only be realized if tree improvement increases such that commercial production becomes feasible. Breeding black walnut for thinner shell, disease resistance, and predictable nut yield are just three of the many agronomically important traits that could be improved using marker-assisted selection (MAS). The goal of this study was to incorporate microsatellite markers to characterize (genotype) 11 cultivars and establish a mapping population from a cross between 'Football' and 'Sparrow' (Ft x Sp). Phenology of these two cultivars coupled with marker characteristics and phylogenetic analysis support the notion of a cross between these two to yield superior hybrids. Of the 51 simple sequence repeat (SSR) markers screened, 23 were polymorphic and useful for genotyping. A synonym was identified, and the final number of cultivars is now 10. Furthermore, seven of the 23 markers were used to identify 63 intraspecific hybrids of the cross 'Football' x 'Sparrow', thereby establishing the first mapping population of black walnut using SSR markers.

KEYWORDS: simple sequence repeat (SSR), microsatellite, black walnut, tree breeding, and marker-assisted selection

This abstract is approved as to form and content

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Chin-Feng Hwang, PhD
Chairperson, Advisory Committee
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INTRODUCTION

Background

The family *Juglandaceae* contains many economically and environmentally important species of hardwood trees. *Juglans sp.* were named for the production of an allelopathic compound, juglone, which acts as an herbicide allowing it to outcompete certain competitive plants. The Persian (English) walnut (*Juglans regia* L.) is the second most valuable nut crop in the world, surpassed only by almond (*Prunus dulcis*). Production of Persian walnuts in 2016 exceeded 600,000 tons in California alone (NASS-USDA 2016). This extremely important and profitable crop has been commercially improved over hundreds of years from wild walnut trees in Central Asia that produce tiny, poor-quality nuts. *Juglans nigra* L., commonly known as the black walnut, is closely related to the Persian walnut, but is unique to North America (Williams 1990). It has a familiar presence in Appalachian and Midwestern landscapes with a native range spanning most of the eastern U.S. and stretching as far west as Kansas, Oklahoma, Texas and Nebraska (Aradhya et al. 2007). Commercial improvement for black walnut only just began in the mid-20th century. In the past, *J. nigra* has been a favored tree of Native American tribes who wisely used the juglone of roots and leaves to treat ailments such as toothaches, headaches, snake bites, ring worm and even as an insecticide against fleas (Michler et al. 2007). Today, black walnut remains a valuable and desirable tree for both timber and nut production, as they display a beautiful veneer and possess prized culinary attributes. Indeed, many people eat them for health reasons despite that its superior nutritional benefits are just becoming known (Gold et al. 2004; Camara and Schlegel

2016). Though the potential to compete in the nut commodity market is great, little has been done to thoughtfully improve the species for commercial production.

Rationale

Breeding. Currently, established orchards/plantations utilize trees grafted to improved cultivars with improved traits, such as those listed in Table 1. Producers and processors pay more for improved varieties, with a thinner shell and larger kernel, as their nut-value can be five times that of wild trees (Coggeshall 2011; Hammons 2016). For instance, Hammons Products Company (Stockton, MO 65785), the world's premier processor and supplier of black walnut for both food and industrial uses, has paid between $0.09 and $0.15/lb ($0.19 to $0.33/kg) for wild-harvested black walnuts in-shell, while improved varieties are purchased at between $0.75 and $0.92/lb ($1.60 and $2.00/kg) (Hammons Products Company 2016). As black walnut is primarily a wild-harvested food crop, it displays annual fluctuations and limitations in yield due to various diseases, abiotic stresses, genetics, and phenology. Black walnuts harvested from the wild or back yards typically have a small nut weight, low percent kernel, thick and fleshy hull, and unpredictable nut-bearing habits. The vast majority of the black walnut industry is based on these wild-harvested, low quality and sporadically produced nuts. This unpredictable nature brings instability to prices and markets from year-to-year.

The black walnut industry has tremendous potential for growth, but is hindered by the lack of superior cultivars. Equipment used in the English walnut industry could be tailored for black walnuts if processing-associated traits were improved, i.e. ease of nut-cracking and hull moisture. Indeed, the best black walnut cultivars currently in use

(including ‘Sparrow’ and ‘Football’ used in this study) are nothing more than promising wild selections that have never benefited from a breeding program of any sort. So it follows that earning potential for producers remains unstable and low as they await higher-yielding varieties with a predictable yield, better nut-cracking quality for efficient harvesting, and disease resistance (Reid 1990; Coggeshall and Woeste 2010).

Use of Markers. Being very large, slow to mature and generally not self-pollinating makes this species a difficult subject for traditional breeding but highlights black walnut as an ideal candidate for purposeful breeding using marker-assisted selection (MAS). However, genotyping resources for breeding in black walnut remain largely undeveloped. Focused efforts in this area could allow more rapid parental identification of offspring to ensure the successful construction of mapping populations, specifically ensuring that only desired offspring are kept from each cross. A mapping population consist of 50-250 progeny grown for the purpose of analyzing parental genotypes and constructing a genetic linkage map for identification of quantitative trait loci (QTL) for future MAS.

Developing DNA markers linked to agronomically important traits can greatly accelerate the breeding process and enable a rapid selection of progeny. Breeding via MAS is economically and environmentally friendly since labor, land and water usage is much less than traditionally required. In short, MAS incorporates biotechnology methods that may include restriction enzymes, DNA primer sequences, gel electrophoresis and many other more modern methods to identify regions on an organism’s genome that are associated with traits of interest (Collard et al. 2004). The ability to do this, in the case of breeding, allows plant breeders to save on resources by keeping only the plants with the

DNA marker of interest. Many methods of marker detection have been developed since the late 1980's, such as:

- Allozymes (Rink et al. 1989) – Enzyme based with low-medium variability and co-dominant, but requires high quantity of DNA (not PCR-based).

- Restriction fragment length polymorphism (RFLP) (Paterson et al. 1988; Fjellstrom and Parfitt 1994, 1995) – High reproducibility detection of co-dominant markers, but time consuming and requires large amounts of high molecular weight DNA.

- Random amplified polymorphic DNA (RAPD) (Nicese et al. 1998) - Low reproducibility detection of dominant markers, but PCR-based amplification of random primers means low quantity of DNA required without needing to know exact target.

- Inter-simple sequence repeat (ISSR) (Potter et al. 2002) - High reproducibility detection of dominant markers, but lower variability. PCR-based means it requires low quantity of DNA and is relatively inexpensive.

- Amplified fragment length polymorphism (AFLP) (Kafkas et al. 2005) - High reproducibility detection of dominant markers with high genomic abundance, but requires high molecular weight DNA.

- Microsatellite or simple sequence repeats (SSR) (Hokanson et al. 1998) - High reproducibility detection of co-dominant markers with high genomic abundance, and is PCR-based so low quantities of DNA required, but expensive.

- Sequence-related amplified polymorphism (SRAP) (Zhang et al. 2016) – High variability and reproducibility detection of dominant markers, PCR-based so low quantities of DNA required and inexpensive.

All have been used with success, but each have their drawbacks in either labor, time, cost or estimation of results. For this study, the microsatellite/SSR markers were utilized due to their high level of allelic variability, relative ease of use, transferability between mapping populations, and being codominant (Pollegioni et al. 2009b; Dogan et al. 2014) (Appendix A). Specifically, SSR markers for black walnut were developed to identify pedigree and parentage, to genotype cultivars, and to characterize the genetic structure, diversity and relationship within natural black walnut populations (Woeste et al. 2002;

Dangl et al. 2005; Robichaud et al. 2006; Victory et al. 2006; Pollegioni et al. 2009a; Dogan et al. 2014). Indeed, a black walnut breeding program in the Midwest incorporated SSR markers to genotype and assess improved cultivars and study pollination dynamics in an orchard setting (Coggeshall and Woeste 2010; Coggeshall 2011; Zhao et al. 2017). Results from Coggeshall (personal communication) were considered when determining the likelihood of a successful open-pollinated cross between our cultivars of interest. Genotyping establishes a fingerprint, verifies identity, which is necessary before crossing individuals. Few, if any, breeding programs are using SSR to develop a mapping population for the purpose of identifying QTL for MAS.

Crossing 'Football' with 'Sparrow'. The important traits for black walnut improvement include nut productivity, high percent kernel, kernel quality, reduced nutshell and hull thickness, tree structural strength, and disease resistance – especially to anthracnose (caused by the fungus *Gnomonia leptostyla*) (Woeste and Beineke 2001; Reid et al. 2004) (Table 1). Anthracnose causes early defoliation of trees at the critical time of nut filling and ripening, and leads to alternate-year bearing of nut crops. The cultivar 'Sparrow' is grown successfully in regions with high anthracnose disease pressure, and in Missouri, is consistently among the top nut producers on a year-to-year basis. However, its nuts ripen unevenly, and it is a weak-wooded tree prone to wind and ice damage, sometimes breaking down under heavy nut loads. The cultivar 'Football' produces quality nuts with a high kernel percentage that ripen evenly, and is a very strong-wooded and hardy tree (Thomas and Reid 2006); nevertheless, it is highly susceptible to anthracnose making it a poor nut producer overall. Both cultivars have a high percent kernel of up to 32% compared to the 7-12% observed at processing stations

in wild trees (Reid et al. 2009) (Table 1). The unique characteristics of 'Sparrow' in disease resistance, and 'Football' in kernel percentage and tree strength render them ideal candidates to understand the genetic and biological mechanisms of anthracnose disease resistance and nut quality. The successful construction of a 'Football' x 'Sparrow' population will increase our knowledge of the genetic determinants of complex agronomical traits.

Goals

The first goal of this project was to identify an informative set of SSR markers for genotyping 11 black walnut cultivars at the Missouri State Fruit Experiment Station (MSFES) in Mountain Grove, MO 65711. These 11 genotypes represent a sampling of highly recommended cultivars from Hammons Products Company. Molecular characterization and phylogenetic analysis allow cultivars displaying diverse and complimentary phenotypes to be analyzed for breeding purposes. The second goal was to develop an F_1 mapping population of 'Football' cross 'Sparrow' ('Football' x 'Sparrow') utilizing the same informative markers for hybrid identification. This study utilized 51 SSR markers for initial genotyping and phylogenetic analysis, and reports the development of an intraspecific F_1 mapping population between 'Football' and 'Sparrow' using the most informative polymorphic SSR markers. Intraspecific hybrids are defined as progeny resulting from a cross between two different cultivars of the same species. When there are 50 – 250 intraspecific hybrids, it is referred to as a mapping population that can be used to genetically analyze cross-over frequency for linkage map construction.

Hypotheses

If at least 10 SSR markers are polymorphic for the 11 cultivars, then a phylogenetic tree can be constructed to aid selection of hybridogenic parents. Given the first hypothesis is supported, if 'Football' and 'Sparrow' cultivars have an above average (or significant) genetic distance as calculated by software (GenAlEx6.5), then a cross between 'Football' and 'Sparrow' (Ft x Sp) is supported. Hybrids will show new combinations of alleles (genotypes) and may display heterosis, traits superior to both parents. If a progeny does not show a new allelic combination based on the parents' genotype, then that progeny is not an intraspecific hybrid.

MATERIALS AND METHODS

Plant Materials

In 2008, eleven accessions of black walnut (Table 1) were planted in a silty loam soil at MSFES, latitude 37.19749, -93.27925 longitude. Average annual precipitation is 46 inches, with no extra irrigation applied. There are three replicates of each genotype for a total of 33 trees in this experimental orchard. The trees were propagated from cuttings that were originally provided from the University of Missouri's Southwest Research Center (MUSRC), Mount Vernon, MO 65712. Trees at the MSFES were planted at 50 foot between-row and 50 foot within-row spacing. For the construction of a mapping population, approximately 850 black walnut seeds were harvested from 'Football' trees at MUSRC during October 2015. 'Football' and 'Sparrow' are in adjacent rows 40 foot apart with 'Sparrow' to the west of 'Football' as the intended pollinator. The walnuts were hulled on-site with a mechanical huller and spread on greenhouse benches to dry for 5 days. Nuts were stratified in the field under 3-4 inches of soil from mid-October to early January, 2016. After 72 days, they were transferred to potting soil in 44-ounce Styrofoam cups for germination in the greenhouse. Three months after germination the seedlings had enough leaves for collection. Pictures of the process from seed collection to leaf collection are available in Appendix B.

DNA Extraction, Amplification, and Fragment Analysis

DNA extraction was carried out as previously described in Adhikari et al. (2014). For PCR, three primers were used in each reaction: a M13-tailed 5'-

TGTAAAACGACGGCCAGT-3' forward primer, a reverse primer, and an M13 sequence with a WellRED fluorescent label (D2-black, D3-green or D4-blue) (Sigma, St. Louis, MO). The fluorescent labeling allowed the pooling of PCR products for use in capillary electrophoresis. Each reaction totaled 10 µl comprised of 0.8 µl nuclease-free water, 0.2 µl $MgCl_2$ (25 mM), 1 µl DNA (10µg/µl), 1 µl WellRED primer (2µM), 1 µl forward primer (0.1 µM) and reverse primer (2 µM), respectively, and 5 µl AmpliTAQ Gold 360 master mix polymerase (ThermoFisher Scientific, St. Louis, MO). Amplification conditions for initial screening of 54 primers (27 markers) on 11 cultivars was according to a touchdown method: 1 cycle for 10 minutes at 94 ^{o}C, 40 cycles of 30 seconds at 92 ^{o}C, 60 seconds at 62-55 ^{o}C, 30 seconds at 72 ^{o}C and 1 cycle of 7 minutes at 72 ^{o}C before being held at 4 ^{o}C. Considering results of the varied annealing temperatures (see raw data in Appendix C-1), the optimum annealing temperature of 60^{o}C was used. Therefore, amplification conditions for a repeat and final screening of the 11 cultivars with an additional 48 primers was: 1 cycle for 10 minutes at 94 ^{o}C, 40 cycles of 30 seconds at 92 ^{o}C, 60 seconds at 60 ^{o}C, 30 seconds at 72 ^{o}C and 1 cycle of 7 minutes at 72 ^{o}C before being held at 4 ^{o}C (see raw data in Appendix C-2). PCR products were confirmed in a 1.5% agarose gel. Only markers with confirmed fragments were further separated by capillary array electrophoresis (CEQ) using a Beckman Coulter CEQ 8000 (Beckman Coulter, Inc., Brea CA). See Appendix D for a sample screen of capillary array electrophoresis from which all results were scored.

SSR Scoring, Analysis and Tree Construction

The PCR products of 51 SSR primer sets (markers) were scored after fragment analysis using the GenomeLab GeXP capillary sequencer software (Beckman Coulter, Inc., Brea, CA). Fragment sizes were double checked for usefulness in genotyping, that is only primer/markers that amplified and were polymorphic across the cultivars. The number, frequency and diversity of alleles, size ranges, genetic distance matrix, and probability of identities were calculated by GenAlEx6.5 (Peakall and Smouse 2006, 2012). The genetic distances were imported to MEGA7 (Kumar et al. 2016) for construction of a dendrogram using the Neighbor-joining method (Saitou and Nei 1987). All marker parameters were calculated according to 10 cultivars belonging to one population.

RESULTS

Dendrogram

The SSR-based genetic distance matrix yielded a dendrogram that placed 'Football' and 'EmmaK' as one group and a second group that included the other nine. The nine were split into two groups, a 'Tomboy' and 'Mintle'/'Mintyle' group and a polyphyletic grouping of the other six (Figure 1). 'Mintyle' and 'Mintle' possessed identical allele sizes at all loci (Table 2), and therefore the calculated genetic distance was "0". The dendrogram reflects this as it placed both cultivars at the end of the same branch. At the onset there were 11 cultivars, however, they are now considered the same cultivar, bringing the count to 10. All other diversity parameters were calculated accordingly. The total branch length of the dendrogram was 185.75 with the shortest distance between two cultivars being 25 ('Kwik-Krop' and 'Vandersloot') and the furthest being 48 ('DOT' and 'Emma K') (Figure 1). 'Football' and 'Sparrow', our cultivars of breeding interest, were placed in two different clades separated by a genetic distance of 45.

Marker Characteristics and Genotypes

Out of 51 SSR primer sets screened, 23 amplified polymorphic with an average of 10.35 alleles per locus and a mean heterozygosity of 0.87 (Table 2 and 3). The number of alleles per marker ranged from a low of 5 (WGA006) to a high of 17 (WGA082) with corresponding high and low probabilities of identity (P_{ID}) being 0.2352 and 0.0093, respectively. The combined P_{ID}, probability that two unrelated individuals share the

same genotype at all loci, for all 23 markers was $2.4x10^{-33}$. Markers WGA024, WGA032, WGA065, and WGA082 each had high allelic richness and showed the most diversity with 10 different heterozygous genotypes for the cultivars (Tables 3 and 4). While four other markers (WGA118, WGA002, WGA074 and WGA086) also reflected 10 unique genotypes with a low P_{ID}, WGA118 was homozygous on 'Mystery', and WGA002, WGA074 and WGA086 were homozygous on two cultivars each, 'Vandersloot' and 'Kwik Krop', 'Hay' and 'Football', and 'Hay' and 'Mystery', respectively (Table 2). WGA006 reflected the least genetic diversity as its 5 alleles yielded only 5 unique genotypes with 9 cultivars sharing its alleles to various extents. This lack of diversity is reflected in the low number of effective alleles (alleles contributing to the diversity due to their frequency in the population) for WGA006 and high probability of identity. However, it is noted that WGA079 had the lowest heterozygosity (0.6) as it flanks 7 alleles containing a narrow size-range (201-211) and was homozygous on four cultivars with three of those possessing the same genotype. The lower P_{ID} of WGA079 is likely due to having a higher number of effective alleles than WGA006, that is there are more alleles distributed evenly across the 10 cultivars (Table 2 and 3).

Marker characteristics for 'Football' and 'Sparrow'

To evaluate the hybridogenic potential of 'Football' x 'Sparrow', further analysis of the 23 markers, using only these two cultivars, was conducted. Their combined number of alleles was 78 (Table 2), and when compared to all cultivars, nine alleles were unique to 'Football' and 13 to 'Sparrow'. Genotypically, 'Football' was homozygous at

7 of 23 loci (H_o = 0.696) while 'Sparrow' was homozygous (H_o = 0.957) at only one locus with six alleles shared between the two (Table 2, labeled underlined and purple). The six shared markers (WGA006, WGA007, WGA009, WGA011, WGA069, WGA79) were at loci that generated a less than average number of alleles, i.e. 5 – 9 and even lower numbers of effective alleles (Table 3). For perspective on the six shared alleles, 'Emma K' and 'Tomboy' have 11 alleles in common, 'Dot' and 'Sparrow' share 11, and 'Kwik Krop' and 'Vandersloot' share 22. These relationships are reflected in the dendrogram (Figure 1). Therefore, it is with good confidence all 23 markers could be used for cultivar identification as well as on progeny for analysis of parentage.

However, not all 23 markers are necessary for F_1 hybrid identification as will be explained in the Discussion. Therefore, four of the 23 SSR markers were not chosen for hybrid identification purposes due to allele sizes being within 3 bp of each other (WGA001, WGA002, WGA024, WGA086), making it difficult to definitively read the peaks of the CEQ chromatogram (Appendix B). Nine others were not selected due to homozygosity or sharing alleles in common (WGA006, WGA007, WGA009, WGA011, WGA053, WGA069, WGA074, WGA079, WGA148) (Table 2). Marker WGA082 was considered useful, however, in practice it did not consistently amplify fragments with allele sizes expected based on the initial genotyping. As a result, nine easily distinguished markers were initially selected for screening intraspecific hybrids (see asterisks on Table 2). Unfortunately, WGA027 and WGA032 displayed null alleles when used for hybrid identification, and/or did not consistently amplify the fragments. Consequently, the number of polymorphic markers used to identify seedlings was reduced to seven. The discriminatory power of these seven markers was re-evaluated for

use in hybrid identification of any two cultivars as well as our two cultivars of interest (Table 4). In other words, the likely hood that these seven markers would yield the same genotypes on any two anonymous individuals, as well as any two known siblings, was calculated.

Mapping Population

Analysis of the power, or ability, for these seven markers to confidently identify hybrids from a cross between trees in this orchard yielded a combined P_{ID} of 3.61×10^{-7}. If used to identify hybrids of two known cultivars, the combined probability that two full siblings would share the same genotype at all loci (P_{Isib}) was 0.0021. Therefore, the seven selected markers were deemed adequate for hybrid identification. Of the 850 seeds collected at MUSRC, 257 (30.2%) germinated. Of the 257 seeds germinated, seven markers confirmed 63 (24.5%) intraspecific hybrids of 'Football' x 'Sparrow'.

DISCUSSION

Eastern black walnut stands on the brink of great market expansion as demand continues to increase, but many of the improved cultivars still lack traits that could foster successful commercial production. Thick shell, low percent kernel, and disease susceptibility are major traits that reduce value or hinder large-scale harvesting and processing. Breeding black walnut using SSR markers is a time and resource conscious way to increase breeding efficiency and will enable a more sustainable and profitable commercial production. At the MSFES, there were 11 black walnut cultivars of breeding interest which had never been genotyped. After fingerprinting them via 23 SSR markers developed for *J. nigra* L. it was observed that 'Mintyle' and 'Mintle' possessed identical alleles at all loci (Table 2). Thus, they are likely synonyms that resulted from a typo or mislabeling during the initial tree establishment. They are now considered one and the same. That each analyzed marker yielded identical fragments, and thus placement at the same point on the phylogenetic tree, reflects the effectiveness of SSR markers to identify closely related or even identical cultivars (Figure 1 and Table 2). Indeed, other studies have used SSR markers to distinguish synonyms (Dangl et al. 2001; Dangl et. al. 2005; Hammers et al. 2017; Zhao et al. 2017). When characterizing genetic relationships, SSR markers had a 30% higher rate of polymorphism than other molecular markers (Dogan et. al. 2014). This gives confidence to conclude that the black walnut orchard at MSFES is comprised of 10 cultivars. For the correct spelling and identification, records at the MUSRC and/or original tree-source will be verified once the synonymous cultivars, which have recently undergone genotyping, are consolidated (Zhao et al. 2017

Comparing results of the orchard population to natural populations, the 23 markers showed a higher mean heterozygosity and similar if not greater allelic richness (total = 239) than similar studies (Woeste 2002; Victory et al 2006; Pollegioni et al. 2009b; Ruiz-Garcia et al. 2011; Vahdati et al. 2014). The lower heterozygosity of WGA006 and WGA079 mirror previous studies on natural populations by Robichaud et al. (2006) and Victory et al. (2006), where combined with other markers, they still contributed to a unique identity. Though each had a relatively low number of effective alleles (Ne), in this study they were easily distinguished and collectively added discriminatory power (Table 3). Therefore, the 23 polymorphic markers were considered useful for aiding selection of the best breeding combinations as well as for confirming and genotyping their F_1 progeny. Clearly 23 markers are not necessary to uniquely identify a cultivar as many studies have successfully incorporated fewer for similar purposes (Pollegioni et al. 2009b; Karimi et al. 2010; Zhao et al. 2017), but do indicate the potential of particular cultivars in this orchard to yield progeny having good hybrid vigor. For instance, when evaluating any two parents' F_1 progeny, the best 10 markers from the 23 may be chosen. For example, if one were interested in crossing 'Kwik-Krop' with 'Vandersloot' then we would not select the seven markers where they shared at least ¾ of alleles in common. By this standard, there are still a potential of 16 markers that could be used, however for financial reasons 10 would suffice.

When identifying parents and hybrids of 'Football' and 'Sparrow, selection of markers that consistently showed clear bands resulted in seven markers that combined for 27 alleles and a probability of identity for full-sibs (P_{Isib}) equal to 0.0021. The combined P_{ID} and P_{Isib} calculated for just seven markers used in this study (Table 4) is comparable

to the combined unbiased P_{ID} and P_{Isib} of Robichaud et al. (2006) using 14 microsatellites. And on average, the P_{ID} is lower than Dangl et al. (2005) using 12 microsatellites on *J. regia* L. accessions. These data indicate the power of the markers to identify intraspecific hybrids. The diversity of this orchard population is likely due to decades of previous tree improvement efforts and the geographical origin of the source trees, which ultimately came from private growers of the greater Midwest via Hammons Products Company. In summary, the allelic richness, low percentage of shared alleles, and high heterozygosity of 'Football' and 'Sparrow' indicate their hybridogenic potential to produce superior progeny.

Generally, the long branch arms of the dendrogram support the notion that cultivars in this orchard population sharing a recent node (ancestor), such as 'Hay' and 'Vandersloot', may even provide good breeding potential if their traits are complimentary. Due to the complimentary display of phenotypic/morphological traits (Table 1) by 'Football' and 'Sparrow', they were considered heterotic parents, able to yield superior hybrids in development of a mapping population. However, diverse morphology alone is not a good indicator of genetic distance in black walnut, nor is genetic distance alone a pre-requisite for favorable crossing. Combining phenotypic observations and genetic diversity parameters, however, offer good support of heterosis. For our goals, one observes that 'Sparrow' is nested in a clade that doesn't share a common node (ancestor) with 'Football' until the base of the dendrogram and is separated by 45 genetic distance units, thus supporting a heterotic cross.

Evaluation of a hybrid mapping population from a wind-pollinated cross is extremely difficult, even in an orchard population, and takes much time. The use of SSR

markers accelerates the process by verifying progeny within months instead of years. Coggeshall (personal communication) used SSR markers to show pollination patterns in an orchard coming from many trees surrounding a selected female, however in that study a large majority of the hybrid progeny came from the tree to the immediate west, having nine days of overlapping pollen shed with female receptivity. Producing hybrids between our parents of interest is probable, as 'Sparrow', the intended male, is planted adjacent to 'Football' on the west. Ensuring selection of 'Football' as the female parent is not difficult since the walnuts are visibly larger and display the characteristic shape for which they are named.

The percentage of intraspecific hybrids we obtained (23.7%) is not uncommon in natural tree stands, but one might expect a higher success rate for an orchard population. This may be due in part to the dichogamous nature of black walnuts or the wind direction during the time of reproductive compatibility. With at least a week difference in leafing date, 'Football' being earlier and protanderous (catkins flush first), pollination by the protogynous 'Sparrow' is certainly possible (Reid et al. 2004). While black walnut can self-pollinate, it predominantly outcrosses (Rink et al. 1989), and has been observed to have high pollen flow in natural populations as well as orchard stands (Victory et al. 2006; Coggeshall 2011). The abundance of pollen flow means more competition, even for an adjacent pollinator in the orchard. Moreover, a brief review of the wind patterns, within the potential walnut pollen production time of March-June, in Mt. Vernon, MO revealed an ideal wind direction (out of west) only 14.3% of the time. Other possible causes of low germination percentage include pre-mature abscission due to tree-shaking, brevity of cold-treatment where the typical time in ground is 90-120 days and this study

cold-treated for ~75 days, having a warmer than usual December, or possibly over-drying before cold-treatment (Kapoor et al. 2010; Flores et al., 2017). Premature abscission and over-drying may be related in that nuts that fall naturally have a lower internal moisture and do not succumb to dry-stress, whereas nuts collected after shaking do not survive as long in dry conditions. Therefore, waiting for natural abscission, or less time drying before stratification may provide a higher germination percentage.

Other strategies may be employed to increase chances that seeds are hybrids of 'Football' x 'Sparrow', such as collecting within a smaller radius of the 'Football' trunk as opposed to edge of the crown, using middle trees as opposed to the orchard perimeter, or even incorporating stratification strategies to increase the germination rate (Flores et al. 2017). An ideal mapping population size for establishing a framework genetic map is about 200 progenies (Hammers et al. 2017). Seed collection will be repeated using strategic methods to improve the chances of collecting 'Sparrow' pollinated 'Football' nuts until a mapping population of ~200 progenies are identified using the seven polymorphic SSR markers noted in Table 4. Presented herein are the first intraspecific hybrids of a wind-pollinated cross of black walnut verified using SSR markers.

CONCLUSION

Of 51 SSR-markers screened against the 10 cultivars at the MSFES, 23 showed polymorphism thereby establishing the tree's genetic identity. Additionally, with a genetic fingerprint on record, collected seeds that germinated the next season were analyzed for parentage. Eight-hundred and fifty seeds of wind-pollinated 'Football' were collected from the orchard floor and evaluated with 7 SSR markers for intraspecific hybridization of 'Football' x 'Sparrow'. Sixty-three F_1 seedlings were true hybrids. More research is needed to optimize germination results for the expansion of this mapping population. The markers utilized herein provided a genotype for 10 cultivars (one confirmed synonym) and have shown to expedite development of a mapping population, which coupled with other markers, may allow linkage map construction for future identification of QTLs to be used in MAS. This project is a first step toward meeting the demand for improved black walnut trees available for commercial production. It is the first reported development of a mapping population using SSR markers. More projects of this kind are needed for eastern black walnut to successfully meet the growing demand.

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TABLES

Table 1. Comparison of phenotypical and agricultural traits of interest for 11 black walnut (*J. nigra*) cultivars at the Missouri State Fruit Experiment Station, Mountain Grove, MO (Andy Thomas of MUSRC personal communication, Reid et al., 2004, 2009).

Cultivar	% Kernel	Nut wt. (g)	Hull	Anthracnose susceptibility	Wood strength	Spur-fruiting	Alter-nate bearing	Flower type
Dot	23	NR	NR	NR	Good		H	
Mintyle	NR	NR	Thin/dry	NR	Good		H	
Mintle	"	"	"	"	"			
Emma K	29.5	19	NR	M	Good	Yes	H	G
Tomboy	27	22	Thin/wet	L	Good	No	M	G
Vandersloot	24		Thick/wet		Good			
Kwik-Krop	31	17	Thick/wet	M	Good	Yes	H	G
Hay	32	22	medium	L	Good	Yes	M	
Mystery	19		Thick	NR	Good	NR	M	
Sparrow	**32**	**19**	**Thin/ dry**	**L**	**Weak**	**No**	**L**	**G**
Football	**29**	**22**	**Thick/wet**	**H**	**Strong**	**Yes**	**H**	**A**

H = high, M = medium, L = low
G = protogynous (female flowers first), A = protandrous (male flowers first)
NR = not recorded NA = not available

Table 2. Genetic diversity and allele sizes (bp) for 23 SSR loci on 11 cultivars. H*o* = observed heterozygosity.

Cultivar	WGA001	WGA002	WGA005*	WGA006	WGA007	WGA009	WGA011	WGA024	WGA027*	WGA032*	WGA053	WGA054*
Dot	190 191	136 152	219 221	143 143	214 226	246 246	211 226	248 259	224 230	193 201	183 196	202 206
Mintyle	182 184	138 140	204 215	143 161	216 226	244 257	211 226	239 246	216 224	188 199	177 183	206 209
Mintle	182 184	138 140	204 215	143 161	216 226	244 257	211 226	239 246	216 224	188 199	177 183	206 209
EmmaK	186 187	122 150	208 225	143 157	214 219	244 250	211 221	246 248	220 226	178 192	190 190	203 213
Tomboy	186 187	140 173	219 221	143 161	212 216	236 250	211 226	233 246	224 230	192 203	183 194	201 211
Vandersloot	184 186	147 147	212 231	143 145	214 214	232 246	211 232	245 252	214 232	172 186	183 200	201 204
KwikKrop	184 190	124 124	202 213	143 145	214 217	232 248	211 230	246 252	214 230	184 186	183 196	201 206
Hay	173 182	119 167	206 212	143 143	214 216	246 276	211 228	244 250	231 235	176 187	183 183	201 204
Mystery	186 188	122 174	215 233	143 157	214 214	232 246	211 226	244 249	220 230	197 199	198 201	209 211
Sparrow	180 190	142 151	182 184	143 151	214 227	246 254	211 211	240 244	230 232	170 184	199 201	201 210
Football	181 182	150 177	206 216	143 143	214 *214*	246 257	211 226	243 245	214 224	192 205	196 196	204 208

	WGA065*	WGA069	WGA074	WGA076*	WGA079	WGA082	WGA086	WGA097*	WGA118*	WGA148	WGA276*	H*o*
Dot	166 168	175 177	183 204	233 233	201 207	200 219	221 229	167 173	222 226	407 407	153 170	0.826
Mintyle	168 172	169 171	183 185	229 233	206 208	196 210	227 231	155 159	221 229	404 408	151 166	1
Mintle	168 172	169 171	183 185	229 233	206 208	196 210	227 231	155 159	221 229	404 408	151 166	
EmmaK	160 162	179 181	187 189	235 237	210 210	163 189	228 230	161 161	235 241	402 422	166 174	0.87
Tomboy	162 164	175 177	179 185	233 237	206 209	171 202	220 222	161 163	227 231	403 404	164 166	1
Vandersloot	135 142	177 179	173 195	235 239	210 210	161 220	220 224	161 163	217 221	403 407	153 153	0.826
KwikKrop	135 168	175 179	195 211	235 237	210 210	208 220	221 242	157 163	217 227	403 407	153 158	0.913
Hay	135 172	177 179	173 173	235 237	210 211	161 177	221 221	160 162	236 246	403 407	151 153	0.826
Mystery	139 185	175 179	196 198	229 233	207 210	171 194	231 231	156 160	229 229	403 403	165 176	0.826
Sparrow	142 150	173 175	173 218	233 243	207 210	175 184	226 248	155 163	222 228	404 408	153 171	0.957
Football	170 172	175 179	193 193	237 237	207 207	207 218	227 229	161 169	223 239	406 406	164 166	0.696

* selected for screening hybrids of 'Football' x 'Sparrow'. Underlined/purple numbers are alleles shared between 'Sparrow' and 'Football'.

Table 3. Diversity of 23 SSR markers in 10 black walnut cultivars.

Locus	Size range	N_g	N_a	N_e	H_e	H_o	P_{ID}
WGA001	173-191	9	10	7.69	0.87	1.0	0.0306
WGA002	119-177	10	15	13.33	0.93	0.80	0.0107
WGA005	182-233	9	15	13.33	0.93	1.00	0.0107
WGA006	143-161	5	5	2.20	0.55	0.70	0.2352
WGA007	212-227	7	7	2.90	0.66	0.70	0.1459
WGA009	232-276	9	9	5.41	0.82	0.90	0.0526
WGA011	211-232	6	6	2.67	0.63	0.90	0.1858
WGA024	233-259	10	12	9.09	0.89	1.00	0.0218
WGA027	214-235	9	9	6.45	0.85	1.00	0.0418
WGA032	170-205	10	15	12.50	0.92	1.00	0.0119
WGA053	177-201	9	9	5.13	0.81	0.70	0.0592
WGA054	201-213	9	10	7.14	0.86	1.00	0.0341
WGA065	135-185	10	12	9.52	0.90	1.00	0.0203
WGA069	169-181	6	7	4.35	0.77	1.00	0.0880
WGA074	173-218	10	13	10.00	0.90	0.80	0.0180
WGA076	229-243	7	6	4.26	0.77	0.80	0.0925
WGA079	201-211	6	7	3.51	0.72	0.60	0.1174
WGA082	161-220	10	17	14.29	0.93	0.90	0.0093
WGA086	220-248	10	12	9.09	0.89	0.80	0.0218
WGA097	155-173	9	11	7.14	0.86	0.90	0.0335
WGA118	217-246	10	14	11.76	0.92	0.90	0.0135
WGA148	402-422	7	7	5.00	0.80	0.70	0.0673
WGA276	151-176	9	10	6.06	0.84	0.90	0.0445
Average	-	8.5	10	7.51	0.82	0.87	0.059

N_g = number of different genotypes

N_a = number of different alleles

N_e = number of effective alleles

H_e = expected heterozygosity

H_o = observed heterozygosity

P_{ID} = probability of identity, that two individuals will share the same genotype at a locus

Table 4. Probabilities of identity for the 7 SSR markers used to screen 257 potential progeny of 'Football' x 'Sparrow'.

Locus	P_{ID}	P_{Isib}	N_a
WGA005	0.109375	0.4023438	4
WGA054	0.109375	0.4023438	4
WGA065	0.109375	0.4023438	4
WGA076	0.2109375	0.4902344	3
WGA097	0.109375	0.4023438	4
WGA118	0.109375	0.4023438	4
WGA276	0.109375	0.4023438	4
Combined	3.6113E-07	0.0020796	27

P_{Isib} = Probability of identity, that two full-sibs would share the same genotype for that marker.

Cultivar	Dot	Mintyle	Mintle	Emma K	Tom boy	Vander sloot	Kwik-Krop	Hay	Mystery	Spar-row	Foot ball
Dot	0										
Mintyle	38	0									
Mintle	38	0	0								
Emma K	48	44	44	0							
Tomboy	37	32	32	37	0						
Vander-sloot	41	45	45	40	40	0					
Kwik-Krop	37	41	41	40	37	25	0				
Hay	38	41	41	44	40	30	34	0			
Mystery	41	38	38	42	39	40	40	42	0		
Sparrow	36	40	40	44	38	36	36	39	38	0	
Football	41	44	44	44	42	45	44	43	45	45	0

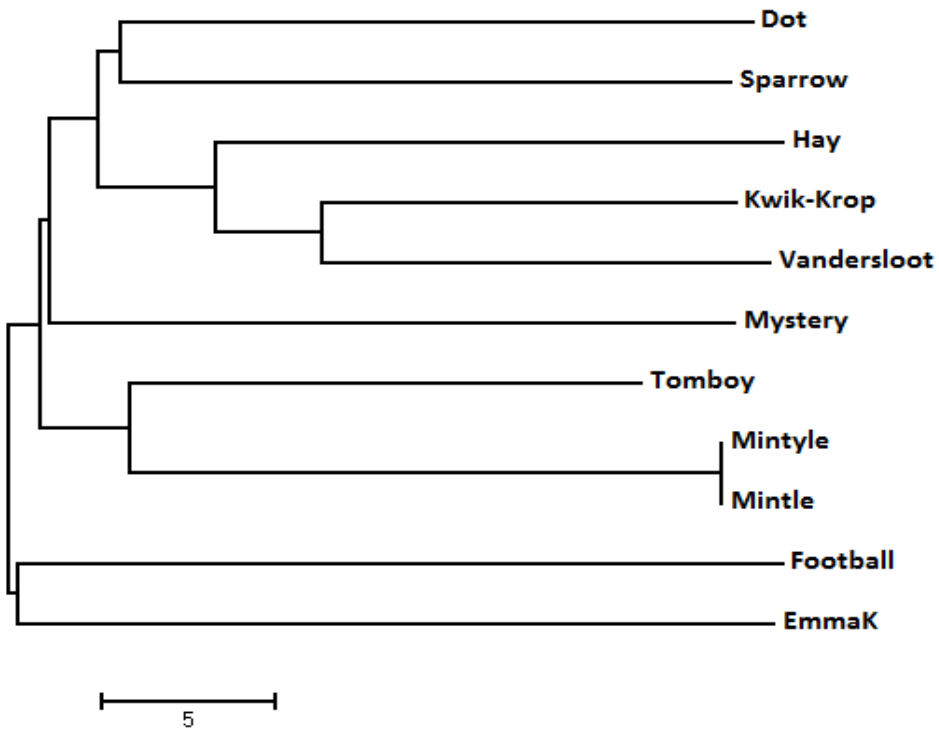
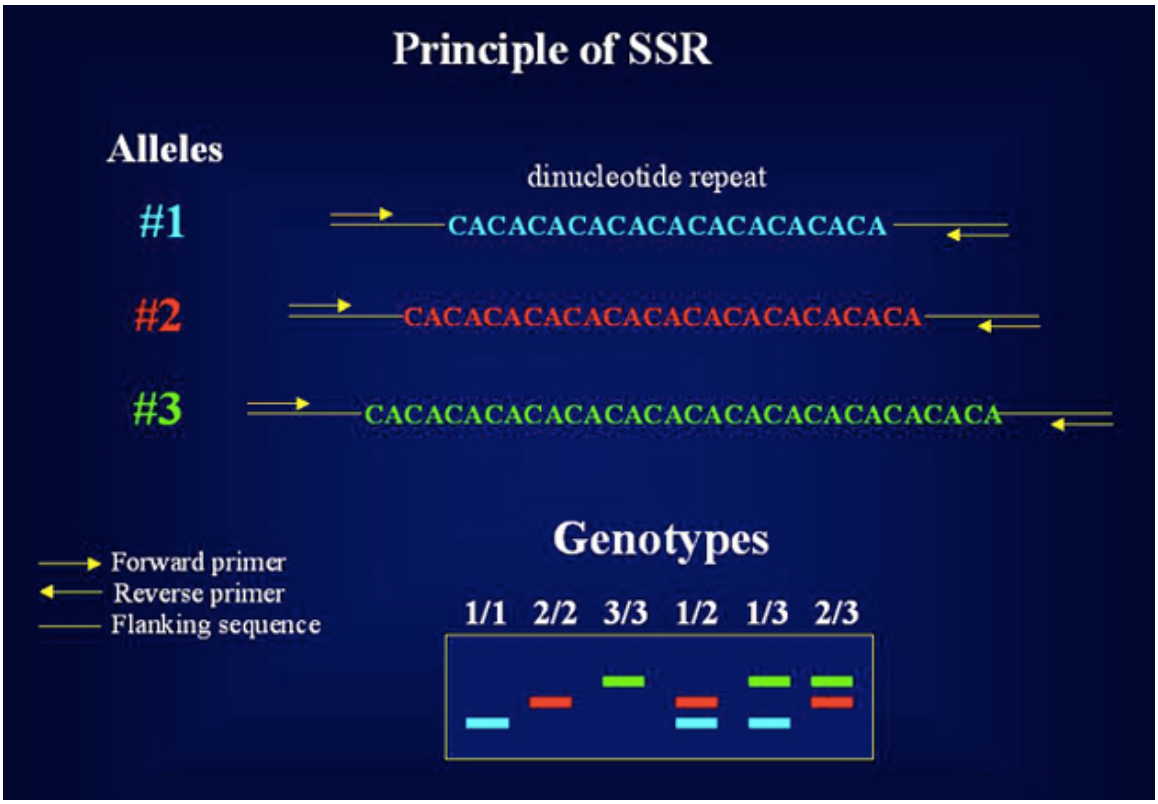


Figure 1. Genetic distances (top) for 11 eastern black walnut cultivars and selections based on 23 SSR markers. Dendrogram (bottom) based on same genetic distance units using the Neighbor-joining method in MEGA7. Total branch length = 185.75.

APPENDICES

Appendix A. Principle of SSR



A diagrammatic explanation of the principle of SSR marker identification. Legend for primer/marker location in reference to the SSR $(CA)_n$ is in lower left corner. Alleles are variations of a gene within a population. In this case, the variable repeat is the dinucleotide "CA". The Genotypes at bottom is he resultant gel pattern of the amplicons after DNA isolation, PCR, and separation by gel electrophoresis. http://www.cdfd.org.in/SILKSAT/index.php?f=protocol_ssr

Appendix B. Workflow in the Field

Collect walnuts from floor (A). Feed the walnuts into the bin on top that contains a chute leading to a spinning tire that causes the walnut to grind against the rebar (B). This pushes the walnut out of a spout on the side where a bucket receives the hulled walnut.

A.  B. 

C. 

D. 

 E.

Spread walnuts in greenhouse to dry for a week (C). Then place in ground for 90-120 days for cold-treatment (D). Plant in 44 oz. cups in greenhouse for germination (E).

Appendix C-1. Raw Data from Initial Primer Screening

Results of the initial black walnut primer screening amplified at 55°-58°C for primers WLLC001-054. Twenty-seven markers from published literature against 11 cultivars of the orchard at MSFES.

Primer numbers	**WLLC 001+002**	**WLLC 003+004**	**WLLC 005+006**	**WLLC 007+008**	**WLLC 09+010**	**WLLC 011+012**	**WLLC 013+014**
Marker name	**WGA001**	**WGA004**	**WGA009**	**WGA069**	**WGA089**	**WGA118**	**WGA202**
Mintle	101(s)/ 199/201	143/266/ 272	264/276	187/189	209/224	(239/240)/ (245/247)	242/270/ 281
Mintyle	100(s)/ 199/200	143/266/ 272	263/276	187/189	209/223	239/237/247 /245	242/270/ 281
Tomboy	202/204/ 206	143/266/ 268	255/269	193/195/ 197	205/213	245/243/249 /247	243/279/ 283
Dot	207/208	143/265/ 269	264	194/196	209/228	242/246/244	282/~247/ ~253
Kwik-Krop	201/208	143/263/ 265	251/268	124/193/ 197	~228	235/245	238/271/ 285
Vader sloot	100(s)/ 201/203	143/259/ 265	251/265	193/195/ 197	211/~228	235/239	~253/271/ 279
Emma K	202/204/ 206	261/263	263/269	197/199	207/217	(251/253)/(2 57/259)	243/267/ 275
Mystery	202/204/ 206	143/(265/ 267)	249/264	193/197	(229/231)=231	243/247	~246/270/ 279
Hay	191/200	143/265/ 267(one)	250/278	195/197(o ne)	222/228	240/249	~254/~265/ 271/281
Football	196/198	143/265/ 267(one)	263/271	193/197	223/230	~241/~257	243/252/ 275/281
Sparrow	196/198/ 208	143/265/ 267(one)	265/277	193	222/231	239/245	242/244/ 269/279

ND = No data

Appendix C-1. Raw Data from Initial Primer Screening- continued

Results of the initial black walnut primer screening amplified at 55°-58°C for primers WLLC001-054. Twenty-seven markers from published literature against 11 cultivars of the orchard at MSFES.

Primer number	WLLC 015+016	WLLC 017+018	WLLC 019+020	WLLC 021+022	WLLC 023+024	WLLC 025+026	WLLC 027+028
Primer name	WGA225	WGA276	WGA321	WGA331	WGA332	WGA349	WGA376
Mintle	(217/219)	169/183	254/264	200/202/ 327	250/252	256~274/ 277	238
Mintyle	(217/ 219)	169/183	254/264	199/201/ 327	250/252	256~274/ 277	238
Tomboy	218/219	181/183	256/264	208/224/ 325	252/254	258/262/278 /281/~292	238
Dot	(218/219)/ (225/226)	171/188	(271/273/2 75)=273	(206/208)/ 327	250/252	250/256/287	238
Kwik-Krop	(212/213)	171/175	256/264	200/208/ 324	(250/252)	256/268/275 /282	238
Vader sloot	(212/213)/ (223/224)	171	256/270	199/212/32 4/331	250/252	256/268/282 /275/290	238
Emma Kay	(217/219)/ (221/222)	183/192	262/264	(195/196)/ ~216/325	248/252	250/258/295 /275/279	238
Mystery	(216/217)/ (223/224)	183/194	262/266	210/212/ 325	(246/248)	250/260/273 /284	238
Hay	(212/213)/ (218/219)	169/171	252/256	204/212/32 5/331	250/252	258/284/268 /289/274	238
Football	(212/213)/ (218/219)	181/183	256/272	(208/206)/ 325	(248/250)	~255/~272/~ 291	238
Sparrow	(217/219)	171/189	256/264	195/210/ 325	(248/250)	~257/~276/~ 291/~308	238

ND = No data

Appendix C-1. Raw Data from Initial Primer Screening- continued

Results of the initial black walnut primer screening amplified at 55°-58°C for primers WLLC001-054. Twenty-seven markers from published literature against 11 cultivars of the orchard at MSFES.

primer number	WLLC 029+030	WLLC 031+032	WLLC 033+034	WLLC 035+036	WLLC 037+038	WLLC 039+040	WLLC 041+042
Primer name	**WGA005**	**WGA027**	**WGA032**	**WGA071**	**WGA072**	**WGA076**	**WGA090**
Mintle	223/~233	235/242	(204/206)/ (215/217)	~231	167/174	248/251	155/164/- 181/187
Mintyle	222/233	235/242	(204/206)/ (215/217)	~230	167/173	247/251	155/163/- 181/187
Tomboy	237/239	~242/ ~248	210/221	~223/ 230	169	251/255	164/177/187
Dot	237/239/ (~297-301)	113(s)/ 242/248	(208/211)/ (217/219)	229/ 233	169/172	251	181/183
Kwik-Krop	220/231	233/248	200/202/ 204	~233	169	253/255	164/168/- 185/195
Vader sloot	~231/~249	233/~251	190/204	231	169/182	253/257	168/176/178 /193/195
Emma K	~226/~239/ ~243	~238/ ~244	196/210	117/23 1/233	167/169	253/255	155/-174/- 181
Mystery	~233/~251	~238/ ~248	212/215/ 217	118/ 229/231	169/176	247/251	172/195
Hay	224/230	~249/ ~253	194/204	231	167/169	253/255	178/180
Football	224/234	232/242	(208/210)/ (220/222)	229	169	255	176/197
Sparrow	(200/199)/ (202/201)	246/248/ 249	187/202	232	169	251/261	174/176/185

ND = No data

Appendix C-1. Raw Data from Initial Primer Screening- continued

Results of the initial black walnut primer screening amplified at 55°-58°C for primers WLLC001-054. Twenty-seven markers from published literature against 11 cultivars of the orchard at MSFES.

primer number	WLLC 043+044	WLLC 045+046	WLLC 047+048	WLLC 049+050	WLLC 051+052	WLLC 053+054
Primer name	WGA006	WGA024	WGA079	WGA082	WGA086	WGA097
Mintle	169/179	257/264	224/226	~214/ ~228	~245/~249	173/177
Mintyle	169/179	257/264	224/226	~214/ ~228	~245/~249	173/177
Tomboy	161/179	251/264	224/227	189/220	238/240	179/181
Dot	161/161	266/277	219/225	~218/ ~227	~239/~247	185/191
Kwik-Krop	161/163	264/270	228/228	~226/ ~238	237/239/ ~260	175/181
Vader sloot	161/163	262/270	228/228	179/~238	238/242	179/181
Emma K	161/175	244/266	118/228	181/207	246/248	179/179
Mystery	161/175	262/267	225/228	189/~212	249/249	174/178
Hay	161/161	262/268	228/229	179/195	239/239	178/180
Football	161/161	261/263	225/225	236/~225	245/247	179/187
Sparrow	161/169	258/262	225/228	193/202	244/246	173/181

ND = No data

Appendix C-2. Raw Data from Initial Primer Screening

Results of the black walnut primer screening for primers WLLC055-102. Fifty-one markers amplified at 60°C on 11 cultivars of the orchard at MSFES.

primer number	WLLC 55+56	WLLC 57+58	WLLC 59+60	WLLC 61+62	WLLC 63+64	WLLC 65+66	WLLC 67+68
Primer name	**WGA02**	**WGA07**	**WGA011**	**WGA017**	**WGA033**	**WGA033**	**WGA042**
Mintle	156/158	234/244	229/~244	221	171/196, 215	229/230	251/258
Mintyle	156/158	234/244	229/~244	221	171/196, 215	229/230	251/258
Tomboy	158/~191	230/234	229/244	221/222	ND	246/247	250/258
Dot	154/170	232/244	229/244	222	ND	226/228	250/258
Kwik-Krop	142	232/235	229/248	221	ND	232/234	251/258
Vader sloot	165-167 (one)	232	229/250	221	ND	232/234	251/258
Emma K	140/168	232/237	229/239/ 244	221	ND	236/268	250/258
Mystery	140/192	232	229/244	221	171/198	237/239/ 243	251/258
Hay	137/185-194	232/234	229/246	221	197/200	232/234	250/258
Football	168/~195	232	229/244/ 249	221	ND	240	258/261
Sparrow	160/169	232/245	229	222	ND	240/242	260/262

ND = No data

Appendix C-2. Raw Data from Initial Primer Screening- continued

Results of the black walnut primer screening for primers WLLC055-102. Fifty-one markers amplified at 60°C on 11 cultivars of the orchard at MSFES.

primer number	WLLC 69+70	WLLC 71+72	WLLC 73+74	WLLC 75+76	WLLC 77+78	WLLC 79+80	WLLC 81+82
Primer name	**WGA045**	**WGA047**	**WGA053**	**WGA054**	**WGA056**	**WGA058**	**WGA060**
Mintle	ND	(164/166) one?	195/201	224/227	179/181 ,183	222/224	245/250
Mintyle	ND	(164/166) one?	195/201	224/227	179/181 ,183	222/224	243-245
Tomboy	ND	146/148	201/212	219/229	164/207	224/230	243/251
Dot	ND	145/185	201/214	220/224	183/191	222/228	249
Kwik-Krop	ND	166/168	201/214, 218	219/224	170/183	230/232	226/245
Vader sloot	ND	166/168	201/218	219/222	179/183	230/232	226/241
Emma K	230/262, 264	168/170	208	221/239	191/193	224/226	ND
Mystery	ND	170/172	216/219	227/229	164/193	216/232	226/245
Hay	ND	166/168	201	219/222	175/183	224/237	226
Football	230/260	162	214	222/226	177	224/226	ND
Sparrow	249/260	166/168	217/219 (208?)	219/228	170/173 /175	224/226	ND

ND = No data

Appendix C-2. Raw Data from Initial Primer Screening- continued

Results of the black walnut primer screening for primers WLLC055-102. Fifty-one markers amplified at 60°C on 11 cultivars of the orchard at MSFES.

Primer number	WLLC 83+84	WLLC 85+86	WLLC 87+88	WLLC 89+90	WLLC 91+92	WLLC 95+96	WLLC 97+98
Primer name	**WGA065**	**WGA070**	**WGA073**	**WGA074**	**WGA078**	**WGA147**	**WGA148**
Mintle	160	184/186	[250/252] one?	201/203	129/133 196/232	380/381	422/426
Mintyle	160	184/186	[250/252] one?	201/203	119/140	380/381	422/426
Tomboy	160	184/186	252/253	197/203	133/148 196	400/401	421/422
Dot	160	186,188/ 192	[250/251] one?	201/222	129	379	425
Kwik-Krop	153/186	183/193	[252/253] one?	213/229	29/148 196/346	397/399	421/425
Vader sloot	153/160	182/184	250/252	191/213	98/130 148	384/399	421/425
Emma K	178/180	ND	250/253	~205/207	ND	ND	ND
Mystery	157/203	ND	[252/253] one?	214/216	128/148	378/382	421
Hay	153/190	186/188	[248/250] one?	[191/193] one?	100/129 148/232	380/384	421/425
Football	188/190	ND	250/251	~211	ND	ND	ND
Sparrow	160/168	ND	250/251	191/~236	ND	ND	ND

ND = No data

Appendix C-2. Raw Data from Initial Primer Screening- continued

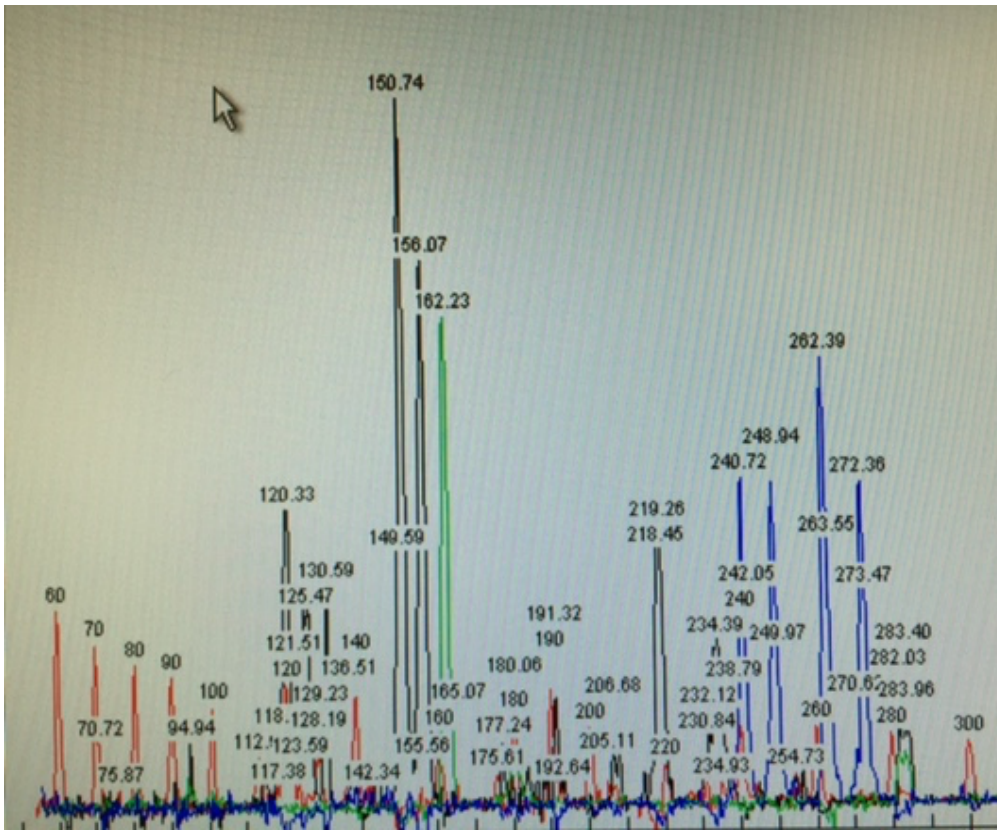
Results of the black walnut primer screening for primers WLLC055-102. Fifty-one markers amplified at 60°C on 11 cultivars of the orchard at MSFES.

Primer number	**WLLC 99+100**	**WLLC 101+102**
Primer name	**WGA069**	**WGA089**
Mintle	187/189	209/224
Mintyle	187/189	209/224
Tomboy	193/195	205/212
Dot	193/195	209/228
Kwik- Krop	193/197	226/228
Vader sloot	195/197	211/228
Emma K	197/199	ND
Mystery	193/197	229/230
Hay	195/197	222/228
Football	193/197	ND
Sparrow	191/193	ND

ND = No data

Appendix D. Sample Screen of Capillary Electrophoresis

Analyzing Alleles



Sample graphic of capillary array electrophoresis data: black (D2), green (D3), blue (D4), and red (internal standard). Number on top of the peak is the length of the allelic fragment. D2 and D4 show the heterozygosity of this allele as 151/156 and 241/249, respectively. D3 shows homozygosity with only one peak at 162 (324 is a different allele as it is too large a difference to be considered the same allele).