GENETIC VARIABILITY AND INHERITANCE OF NUCLEAR MICROSATELLITE MARKERS IN MINNESOTA'S BLACK ASH (*Fraxinus nigra*) AND GREEN ASH (*F. pennsylvanica*) WITH RECOMMENDATIONS FOR THE OPTIMAL SEED SAMPLE SIZE FROM INDIVIDUAL TREES

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Chapter 1: Thesis Introduction

In recent years the emerald ash borer (*Agrilus planipennis* Fairmaire or EAB) has become a threat to all ash species in the United States and Canada. It arrived in North America aboard wooden packing materials from Asia and has spread to two Canadian provinces and 18 states including Minnesota. The EAB larvae tunnel into the cambium to feed, creating serpentine galleries which girdle the tree and eventually kill it (Bauer et al. 2003). Since its arrival EAB has killed tens of millions of ash trees in North America (Emerald Ash Borer 2012).

The genus *Fraxinus*, commonly known as ash, has great economic, ecological, and cultural value in Minnesota. Black (*F. nigra*) and green ash (*F. pennsylvanica*) are found statewide in both urban and rural areas. They are harvested for pulpwood, sawlogs, fuel, and veneer (VanderSchaaf 2012). Their seeds are a source of food for many birds and mammals (Dickerson 2002). Black ash are culturally important for use in basketmaking to several Native American tribes (Benedict and Frelich 2008).

To preserve genetic variation in ash species in light of EAB, an *ex situ* seed bank has been developed in a cooperative effort among several agencies of the United States Department of Agriculture (USDA), the Canadian Forest Service, tribal governments, state agencies (The NPGS Ash Conservation Project 2012), and researchers at the University of Minnesota. *Ex situ* seed banks are used effectively for long-term storage of seeds. They are beneficial as a backup seed source when a species is threatened by an introduced pest and has not yet developed resistance or tolerance (Rajora and Mosseler

2001). This *ex situ* seed bank also presents a valuable resource for investigating genetic diversity in Minnesota's ash populations.

The second chapter of this thesis presents procedures and results for four objectives. First was to determine whether nuclear microsatellite markers derived from other *Fraxinus* species could identify genetic variation in Minnesota's black and green ash. Second was to calculate basic genetic diversity descriptive statistics for several black and green ash families from across Minnesota. Third was to use resampling of a subset of data to estimate the optimal number of seeds to sample from each tree to capture a specified amount of the total genetic diversity. Finally, the fourth objective was to compare seed collections made from the same set of trees in a high seed production year and again in a low seed production year to see whether differences existed in the genetic information captured between years.

The third chapter presents the conclusions of this research, management implications, and future directions for the project.

Chapter 2: Genetic variability and inheritance of nuclear microsatellite markers in Minnesota's black ash (*Fraxinus nigra*) and green ash (*F. pennsylvanica*) with recommendations for the optimal seed sample size from individual trees

Introduction

The genus *Fraxinus*, commonly known as ash, has great ecological, economic, and cultural value in Minnesota. Three ash species are native to Minnesota: white ash, green ash, and black ash. The genus *Fraxinus* belongs to the Oleaceae family which also includes the genera *Olea* (olive), *Syringa* (lilac), *Jasminum* (jasmine), and *Ligustrum* (privet). Thirteen additional *Fraxinus* species are found across the United States (Hardin et al. 2001).

According to 2009 Forest Inventory and Analysis (FIA) data, 792 million black ash (*F. nigra*), 201 million green ash (*F. pennsylvanica*), and 2.7 million white ash (*F. americana*) grow in Minnesota's forests. These numbers indicate that currently we have more ash trees than any other state (The Victim: Trees at Risk 2012). By forest type, ash makes up 49% of the Lowland Hardwoods forest type, 5% of the Northern Hardwoods forest type, and 3% of the Aspen/Balsam poplar forest type (VanderSchaaf 2012). By volume, live ash makes up 7.3% out of all species across all ownerships (Jacobson 2009).

In 2009, 65,400 cords of ash were harvested in Minnesota for pulpwood, sawlogs, fuel, and veneer. This represented 2.4% of all wood harvested in Minnesota in 2009 (VanderSchaaf 2012). The hardness and flexibility of green ash wood make it suitable for historic use in baseball bats and tool handles (Wright and Rauscher 1990). Black ash wood can be easily manipulated into thin strips which is ideal for making snowshoes and in canoe construction (Anderson and Nesom 2006). This quality also makes black ash suitable for basketmaking, which is culturally and economically important to several Native American tribes in eastern North America (Benedict and Frelich 2008).

Black and green ash are found statewide. Green ash grows well on moist, well-drained sites and has been widely planted for shade in urban areas (Kennedy, Jr. 1990). Black ash generally is found on moister sites than green ash and makes up the greatest volume of all ash species in Minnesota (Jacobson 2009). White ash is found mainly in the extreme southeastern part of the state on rich, moist, well-drained soils (Schlesinger 1990). It is a minor species in our state, and it will not be considered further here.

Black ash is an important component of wetlands throughout the North American boreal forest, which provide nesting sites for birds and habitat for other small plant species (Benedict and Frelich 2008). Black ash swamps are associated with the black bear's preferred early spring foods in northeastern Minnesota (Rogers and Allen 1987). Ash seeds are eaten by many birds and mammals including wood ducks, quail, bobwhite, purple finches, pine grosbeaks, wild turkeys, beavers, porcupines, and mice (Dickerson 2002, 2006). Twigs, buds, and foliage provide browse for deer and moose (Wright and Rauscher 1990).

Green ash trees are dioecious and diploid with 46 chromosomes (Wright 1959). Black ash trees are polygamous (Bonner and Karrfalt 2008) with unknown ploidy level and number of chromosomes. Both species are wind pollinated, large, and long-lived (Wallander 2008). Ash fruits are long, winged samaras, each containing one seed and are formed in clusters (Bonner and Karrfalt 2008). In black ash, high seed production occurs every one to eight years, with low seed production in the intervening years (Erdmann

1987). Bonner and Karrfalt (2008) report high seed production in green ash every year but personal observation of Minnesota trees suggests a lower frequency.

In recent years the emerald ash borer (*Agrilus planipennis* Fairmaire or EAB) has become a threat to all ash species in the United States and Canada. It was first discovered in Detroit, MI in 2002, although dendrochronology shows that it was present in the area at least ten years prior (Cappaert 2005). EAB is native to eastern Asia and likely made its way to North America aboard wooden packing materials carried on cargo ships into the port of Detroit. Since its arrival it has spread to 18 states and two provinces: Connecticut, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Massachusetts, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Tennessee, Virginia, West Virginia, and Wisconsin, Ontario, and Quebec. Since its arrival EAB has killed tens of millions of ash trees (Emerald Ash Borer 2012). Spread has been enabled by unintentional transport on infested firewood and nursery stock (Poland and McCullough 2006).

In Minnesota, EAB was first found in St. Paul (located in Ramsey County) in 2009 and has since been found in Hennepin, Houston, and Winona counties (Emerald Ash Borer Prevention, Early Detection, and Rapid Response 2012). As in other states, quarantines have been placed on these counties to limit the movement of ash wood and to slow the spread of EAB outside of the quarantine area.

The adult emerald ash borers feed on ash leaves, then mate and lay eggs on the bark. Larvae hatch within two weeks (Poland and McCullough 2006) and tunnel into the cambium to feed, creating serpentine galleries in the phloem and outer sapwood (Bauer et al. 2003). They complete development over the winter and adults emerge in the spring from D-shaped exit holes in the bark. For reasons that are not yet clear, some individuals take longer to develop and emerge the second spring (Poland and McCullough 2006).

Feeding of the adult EAB on the ash leaves causes no lasting damage to the tree but larval galleries girdle the tree, eventually killing it. EAB has killed green ash, black ash, white ash, and blue ash in both rural and urban areas, and it's highly likely that all North American ash species are susceptible (Poland and McCullough 2006). Larvae have been observed in stems and branches from 2cm to 1.5m in diameter, suggesting that any size tree is vulnerable (Cappaert 2005). Of particular concern in Minnesota is that black ash is an important component of the state's northern wetlands and it is not known how the ecosystem would be affected by widespread mortality.

With the possibility of EAB eliminating most of the ash trees in infested areas, several efforts were initiated to collect ash seeds for an *ex situ* seed bank. *Ex situ* seed banks are effectively used for the long-term storage of orthodox seeds, those that remain viable even when dried to very low moisture content (Thormann et al. 2006). They are beneficial as a backup seed source when a species is threatened by an introduced pest and has not yet developed resistance or tolerance (Rajora and Mosseler 2001).

The National Ash Seed Collection Initiative was begun in 2005 at the United States Department of Agriculture Natural Resources Conservation Service (USDA-NRCS) Rose Lake Plant Materials Center. It has evolved into a cooperative effort among the USDA-NRCS, the USDA-Agricultural Research Service (USDA-ARS), the USDA Forest Service (USDA-FS), the Seeds of Success program (Seeds of Success 2012), the Canadian Forest Service, tribal governments, and state agencies. The goal is to create a comprehensive, well-organized collection of seeds from *Fraxinus* populations (The

NPGS Ash Conservation Project 2012). Accessions are documented in the National Plant Germplasm System (NPGS) Germplasm Resources Information Network (GRIN) database. They are available for use in research and will be made available for restoration once EAB has been brought under control (Karrfalt 2010).

Before EAB arrived in Minnesota, several questions arose: Are genetic markers available to investigate black and green ash? How genetically diverse are these species? How many seeds need to be collected from each tree to represent the genetic information from that family? Do collections made from the same tree in different years capture the same amount of genetic information?

The *ex situ* seed bank represents a valuable resource for investigating these questions. Currently, very little genetic information is available for any of the North American ash species. A search of GenBank, the annotated database of all publicly available DNA sequences (Benson et al. 2012) in June 2012 yielded less than 50 nucleotide sequences each for black, green, or white ash. To put this in perspective, European or common ash (*F. excelsior*) is an economically important species found throughout Europe (Dobrowolska et al. 2011) that has been studied extensively and a similar search in GenBank yielded over 12,000 nucleotide sequences.

Several nuclear microsatellite markers have been identified in *F. excelsior* (Brachet et al. 1999, Lefort et al. 1999) and in pooled phloem samples of green, white, black, blue (*F. quadrangulata*), and Manchurian ash (*F. mandshurica*) (Bai et al. 2011). Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs) are multiple repeats of one to six nucleotides. They are found throughout the genomes of all organisms in both protein-coding and noncoding regions (Toth et al.

2000). If the DNA sequence flanking the microsatellite is conserved among *Fraxinus* species then the markers identified in common ash could be used in black and green ash. Polymorphism among individuals is investigated by PCR (Polymerase Chain Reaction) amplification from genomic DNA using two short primers designed to match the DNA sequence on either side of the microsatellite repeat. Amplification products are resolved using capillary gel electrophoresis to visualize the polymorphism among individuals (Powell et al. 1996).

Other genetic markers identified in *Fraxinus* include RAPDs (Jeandroz et al. 1996), isozymes (Ruedinger et al. 2008), chloroplast PCR-RFLP (Heuertz et al. 2004b), and chloroplast microsatellites (Harbourne et al. 2005), but nuclear microsatellites were the genetic marker of choice for this study for three reasons. First, they are codominant, enabling homozygotes to be distinguished from heterozygotes. Second, microsatellites have sufficiently high mutation rates to provide high allelic diversity, contributing to the possibility of statistically powerful analyses. Third, only a small amount of DNA starting material is needed for PCR (Selkoe and Toonen 2006). Drawbacks to using microsatellite markers include null alleles and inconsistent peak patterns, which can cause difficulty in determining the correct genotype.

This project had four objectives. First was to determine whether nuclear microsatellite markers derived from other *Fraxinus* species could identify genetic variation in Minnesota's black and green ash. Second was to calculate basic genetic diversity descriptive statistics such as number of alleles and observed and expected heterozygosities for several black and green ash families from across Minnesota. Third was to use resampling of a subset of data to estimate the optimal number of seeds to sample from each tree to capture the desired amount of genetic diversity. Fourth was to compare seed collections made from the same set of trees in a high seed production year and again in a low seed production year to see whether a difference existed in the genetic information captured between years.

Materials and Methods

Plant material

A family consists of a maternal parent and the seeds collected from it. Trees were open pollinated therefore the number and genotypes of the paternal trees are unknown. All of the seeds collected from one tree are assumed to be half-sibs.

Ash fruits (samaras) were collected from black and green ash located throughout Minnesota. In this study the fruits are simply called seeds. Debris was removed from each sample, wings were left intact, and seeds were submitted to one of three locations for storage according to collection method. Volunteer collections, i.e. those not collected by University of Minnesota staff, were sent to the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO; population collections consisting of more than 25 trees from an area were sent to the USDA-ARS North Central Regional Plant Introduction Station in Ames, IA; and ecoregion collections, i.e. those containing seeds from fewer than 25 trees, were sent to the USDA Forest Service National Seed Laboratory in Dry Branch, GA. When seeds were sent to Ames or Dry Branch, aliquots were forwarded to Fort Collins. Seeds were stored at -18°C and 30% relative humidity (The NPGS Ash Conservation Project 2012), which is equivalent to equilibrium moisture content of 4.8%-6.3 % (Simpson 1998). Seeds for this study were returned to the authors as needed.

A total of eight different green ash families and nine different black ash families with 31 or 128 seeds per family were used to obtain DNA for genetic analysis (Table 1). Dormant vegetative buds from each maternal parent were also collected to obtain DNA for analysis.

DNA methods

Marker selection

PCR primer sequences for 24 nuclear microsatellite markers were obtained from studies in *Fraxinus excelsior* (Brachet et al. 1999, Lefort et al. 1999, Bai et al. 2011). All 24 markers were tested in PCR in black and green ash by varying the annealing temperature and cycling parameters. Of these, nine were chosen for subsequent use in PCR: FEMSATL4, FEMSATL11, FEMSATL16, FEMSATL19 (Lefort et al. 1999), M2-30a (Brachet et al. 1999), 1502, 7867, 35207, and 43402 (Bai et al. 2011). When necessary, primers were redesigned using Primer3 (Primer3 2009). Five of these markers amplified consistently and reproducibly in black ash (FEMSATL4, FEMSATL11, FEMSATL19, M2-30a, and 7867) and seven markers in green ash (FEMSATL11, FEMSATL16, FEMSATL19, M2-30a, 1502, 35207, and 43402). Primer information is reported in Table 2.

DNA isolation

Maternal DNA was obtained from dormant buds. Bud scales were removed and bud tissue was frozen under liquid nitrogen and ground to a fine powder with a ceramic mortar and pestle. DNA was isolated using a DNA Plant Mini Kit (Qiagen) according to the manufacturer's protocol.

Seed DNA was obtained from embryos. The pericarp was removed by hand and seeds were imbibed in sterile water for 48 hours. Embryos were dissected from the seed using a new razor blade or a sterile scalpel blade, taking care not to include any parts of the endosperm or seed coat. Embryos were ground in a 96-well format using liquid nitrogen and 3mm beads in a TissueLyser (Qiagen). Embryo DNA was isolated using a BioSprint 96 DNA Plant Kit (Qiagen) according to the manufacturer's protocol. DNA concentration and purity were assessed by UV spectrophotometer. DNA was diluted in nuclease-free water to a working concentration of 5ng/ul for use in PCR.

Polymerase chain reaction (PCR)

PCR was performed in an MJ Research PTC-200 thermocycler. Markers FEMSATL4, FEMSATL11, FEMSATL16, FEMSATL19, and M2-30a were amplified using singleplex PCR. Reactions consisted of a total volume of 15µl containing 25ng template DNA, 1X GeneAmp PCR Gold Buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM fluorescently labeled forward primer, 0.2µM reverse primer, and 0.375U AmpliTaq Gold DNA polymerase (Applied Biosystems). In addition, FEMSATL4, FEMSATL11, and M2-30a reactions required the addition of Q Solution (Qiagen) to a 1X final concentration. The PCR profile was: 95°C for 5 min; 35 cycles of 95°C for 30 sec, T_m

for 30 sec, 72°C for 30 sec; and 72°C for 20 min. See Table 2 for specific T_m for each marker.

Markers 1502, 7867, 35207, and 43402 were amplified by singleplex or multiplex PCR. Singleplex reactions consisted of a total volume of 10µl containing 10ng template DNA, 1X GeneAmp PCR Gold Buffer, 1.5mM MgCl2, 0.1mM dNTPs, 0.4µM fluorescently labeled forward primer, 0.2µM reverse primer, and 0.5U AmpliTaq Gold DNA polymerase. Multiplex reactions consisted of the same final concentrations of all components just mentioned with the addition of the second fluorescently labeled forward primer (0.4µM final concentration) and the second reverse primer (0.2µM final concentration). The PCR profile was: 94°C for 5 min; 35 cycles of 94°C for 20 sec, 59°C for 20 sec, 72°C for 30 sec; 8 cycles of 94°C for 30 sec, 53°C for 15 sec, 72°C for 30 sec; and 72°C for 10 min.

In black ash, all PCR reactions were singleplex. In green ash, multiplexes consisted of 7867 amplified with 35207 and 1502 amplified with 43402. Data for 7867 in green ash was not included in the final analysis because of extreme difficulty distinguishing among alleles.

Fragment Analysis

PCR products were diluted and/or pooled together according to fluorescent dye and fragment size range. Pool *a* was a green ash pool consisting of 4µl FEMSATL11, 4µl M2-30a, and 2µl 7867/35207 pcr products; pool *b* was a green ash pool consisting of 2µl FEMSATL19, 6µl FEMSATL 16, and 2µl 1502/43402 pcr products; pool *c* was a black ash pool consisting of 7µl FEMSATL4 and 3µl FEMSATL11 pcr products; and pool *d* was a black ash pool consisting of 1µl FEMSATL19, 1µl 7867, and 7µl M2-30a pcr products. Fluorescently labeled PCR products were detected on an Applied Biosystems 3730xl DNA Analyzer at the University of Illinois Core DNA Sequencing Facility in Urbana, IL. Data was visualized in Peak Scanner v1.0 (Applied Biosystems 2006) and fragment sizes were recorded. Binning (converting raw data into whole numbers representing alleles) was performed manually in Excel.

Statistical Analyses

Descriptive statistics including observed number of alleles (A_O), observed heterozygosity (H_O), and expected heterozygosity (H_E, also known as gene diversity) were calculated per marker for each family using Microsatellite Toolkit (Park 2001, 2008) and Arlequin (Excoffier et al. 2005).

$$H_O = \frac{\#\ of\ heterozygotes}{N}$$

$$H_E = \frac{N}{N-1}(1 - \sum_{i=1}^{k} p_i^2)$$

where N is the number of individuals sampled, k is the number of alleles, and p_i is the frequency of the ith out of k alleles (Nei 1987).

A Chi-square goodness of fit test (Eck and Ryan 2012) was used to compare observed frequencies of the maternally inherited alleles with frequencies expected under Mendelian inheritance. Evidence for null alleles was assessed using Microchecker (Van Oosterhout et al. 2004).

Resampling was performed after Miyamoto et al. (2008) using the program Resampling Stats v4.0 (Simon 2012) on data from two selected families to estimate the optimal number of seeds to sample when a specified amount of genetic diversity is desired. Genotypes were randomly selected without replacement from the pool of seed genotypes from N=1 to 126 (for family MNBA0014 with marker M2-30a) or N=1 to 128 (for family MNBA0115 with marker FEMSATL4). For each resample the number of different alleles present was counted. Resampling was performed 1000 times for each level of N. A 95% confidence interval was calculated in Excel for the mean of the allele count for each 1000 iterations at each level of N.

For the seed collections made from a set of trees in two different years, the Shannon index (H) was calculated using the *vegan*: Community Ecology Package in R (Oksanen et al. 2012) for each collection as a measure of genetic diversity:

$$H = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of different alleles and p_i is the frequency of the ith out of S alleles.

A two-tailed t-test was used to check for significant differences in the Shannon index between collection years after Magurran (1988). The t-value which follows Student's t distribution with df degrees of freedom is calculated as:

$$t = \frac{|H_1 - H_2|}{\sqrt{Var(H_1) + Var(H_2)}}$$

where H_1 is the Shannon index for the first collection year and H_2 is the Shannon index for the second collection year. Variance of the Shannon index ($Var(H)$) is calculated as:

$$Var(H) = \frac{\sum p_i\,(\ln p_i)^2 - \left(\sum p_i \ln p_i\right)^2}{N} + \frac{S-1}{2N^2}$$

and degrees of freedom (*df)* are calculated as:

$$df = \frac{(Var(H_1) + (Var(H_2))^2}{(Var(H_1))^2/_{N_1} + (Var(H_2))^2/_{N_2}}$$

where S is the number of different alleles, p_i is the frequency of the ith out of S alleles, N is the number of individuals in the family, and α=0.05.

Results

Microsatellite markers

Out of 24 nuclear microsatellite markers tested, nine markers worked reliably and reproducibly in black and/or green ash (Table 2). All nine markers were polymorphic, amplifying between two and 42 alleles. The remaining markers did not work well enough to be used further due to poor or nonspecific amplification, monomorphism, or unresolvable amplification products (Table A1).

To improve amplification quality, both primers for marker FEMSATL11 and the forward primer for FEMSATL4 were redesigned.

Families

A total of eight green ash families were genotyped across seven microsatellite markers and nine black ash families were genotyped across five microsatellite markers.

Three green ash families and three black ash families each contained 31 seeds. All other families are over fourfold larger with 128 seeds (Table 3a, 3b).

Null alleles

Three markers showed strong evidence of null alleles in six families. Evidence included a higher number of observed homozygotes than expected and seed genotypes that appeared to have no contribution from the maternal genotype.

First, a higher number of observed homozygotes than expected was identified by Microchecker (Van Oosterhout et al. 2004) in four families in FEMSATL4: JDZBA2-1, JDZBA2-2, MNBA0014, and MNBA0023. The same was observed for marker M2-30a in MNGA0143 and FEMSATL16 in MNGA0365.

Second, the maternal parents in these six families amplified only one allele and each seed fell into one of three categories: 1) it amplified one or two alleles with one being the same size in base pairs as the maternal allele, 2) it amplified one allele but it was *not* the same size as the maternal allele, or 3) no amplification. All three categories combine to form the pattern that would be observed if the maternal parent was heterozygous with one null allele. In the categories above, 1) would be homozygous for the maternal allele or heterozygous with the null allele, 2) would be heterozygous with the null allele, and 3) would be homozygous null.

In the families with suspected null alleles, the null allele was assumed and a coded allele size was used where there was certain to be a null allele, i.e. in category two explained in the previous paragraph. For example, FEMSATL16 allele sizes ranged from 169 to 199 and the null allele was coded as 916. However, with this system all seed genotypes amplifying only one allele that matched the maternal allele were not included in analysis because it could not be determined whether the seed was homozygous or heterozygous with the null allele. Likewise, seeds that showed no amplification were not included in analysis because it could not be determined whether the genotype was homozygous null or the reaction failed (Jessica Petersen, personal communication, 7 May 2012).

Genetic diversity descriptive statistics

Descriptive statistics were calculated for all families regardless of size but to avoid the need for rarefaction, subsequent comparisons of allelic variation and heterozygosity were made using only the large 128-seed families (Table 3a, 3b). When the number of seeds successfully genotyped (*N*) fell below 31 or 128, it was due to the known presence of null alleles, or the sample simply failed due to problems with the PCR reaction, capillary injection, or pipetting.

The observed number of alleles (A_O) varied among markers (Table 3a, 3b). For example, in green ash, A_O varied from five alleles in 35207 to 40 in FEMSATL11. In black ash, A_O varied from two alleles in FEMSATL11 to 42 in FEMSATL4.

The observed number of alleles (A_O) varied between species for the same marker. For example, FEMSATL11 was the most polymorphic in green ash with 40 alleles but was the least polymorphic in black ash with only two alleles.

For some markers, A_O varied among families of the same species. For example, among the large green ash families, A_O for FEMSATL11 varied from 18 in MNGA0143 to 29 in MNGA0365. Among the large black ash families, A_O for M2-30a varied between 15 in MNBA0014 and 28 in MNBA0144.

For other markers, A_O remained constant among families. For example, FEMSATL19 showed seven alleles in all large green ash families and FEMSATL11 showed two alleles in all black ash families.

Marker mean observed heterozygosity (marker mean H_O) varied among markers (Table 3a, 3b). For example in green ash, marker mean H_O varied from 0.327 in 35207 to 0.868 in FEMSATL11. In black ash, marker mean H_O varied from 0.405 in FEMSATL19 to 0.903 in FEMSATL4.

Marker mean H_O varied between species for the same marker. For example, marker mean H_O in both FEMSATL11 and FEMSATL19 was twice as high in green ash as it was in black ash. In M2-30a marker mean H_O was higher in black ash than in green ash.

Observed heterozygosity (H_O) varied widely among families for all markers. A minimum difference of 10% exists between the lowest and the highest H_O for every marker, in every family, in both species.

Global H_O was calculated for each marker by considering all genotypes within a species as one "population" (Table 4). In green ash, markers ranged in global H_O from 0.276 to 0.884 and in black ash, markers ranged in global H_O from 0.478 to 0.868. Global H_O was higher in green ash for all three markers used in both species.

Observed heterozygosities (H_O) of 1.0 occur in the families assumed to have null alleles. The genotyping system explained above eliminated the genotypes that were homozygous, consequently raising the proportion of heterozygotes (H_O) to the maximum level possible.

Mendelian inheritance

Mendelian inheritance was evaluated for each marker in all families regardless of size. For each family with each marker, counts of seeds displaying maternal alleles were compared with counts expected under Mendelian inheritance using a Chi-square goodness of fit test (Table 5a, 5b).

Maternal parents were genotyped at each marker by PCR amplification of DNA from dormant bud tissue. Paternal parents and genotypes were unknown. PCR-derived maternal genotypes were unavailable for trees MNBA0023 and JDZBA2-1 with marker M2-30a and tree JDZBA2-1 with FEMSATL 4 because multiple PCR attempts failed to produce products. PCR-derived maternal genotypes were unavailable for tree MNGA0344 for all markers because maternal tissue was unavailable. For these families maternal genotypes were inferred from the seed genotypes.

For a heterozygous maternal parent the expectation is that each allele is inherited by half of the seeds, and for a homozygous maternal parent it is expected that each seed will inherit one copy of the maternal allele. Chi-square tests were not possible for families in which the maternal parent was homozygous, because when only one category is present the chi-square statistic is undefined. Chi-square values ranged from 0 to 26.791 in green ash and from 0 to 31.154 in green ash.

Seed genotypes were identified as unambiguous or ambiguous regarding maternal contribution (Table 5a, 5b). An unambiguous genotype is one in which the maternal contribution can be identified and an ambiguous genotype is one in which the maternal contribution cannot be identified (for example when the seed has the same heterozygous genotype as the maternal parent). Chi-square values were calculated using only unambiguous genotypes.

In all families, seeds shared at least one allele with the maternal parent in every case but three. One seed from family MNGA0143 in marker M2-30a, one seed from JDZBA1-1 in FEMSATL 4, and one seed from MNBA0001 in FEMSATL 4 shared no alleles with their respective maternal parent. However, these three individual seeds *did* share a maternal allele in all other markers. This discrepancy was possibly due to contamination of the DNA template leading to amplification of an undesired product or a botched capillary injection leading to misrepresentation of the product size. However, the quality of the electropherogram raw data was consistent with other samples. These three samples could not be used for Mendelian inheritance analysis but were included in all other analyses.

Number of seeds to capture genetic diversity

Genotypic data from two families was selected to determine the optimal number of seeds to represent the genetic diversity. One family displayed a moderate number of observed alleles and the other family displayed the maximum number of alleles (Figure 1a, 2a).

Selected families were MNBA0014 in marker M2-30a (containing 15 alleles) and MNBA0115 in marker FEMSATL4 (containing 32 alleles). Resampling was performed by randomly selecting individual genotypes without replacement from the pool of seed genotypes from N=1 to 126 (family MNBA0014) or N=1 to 128 (family MNBA0115). For each new resample the number of different alleles present was counted (Figure 1b, 2b). Resampling was performed 1000 times for each level of N.

The mean number of alleles was calculated for each 1000 replications at each level of N (Figure 1c, 2c) along with 95% confidence intervals for the mean (Table 6a, 6b). By combining the mean number of alleles from resampling with the 95% confidence intervals, the number of seeds that capture 80%, 85%, 90%, and 95% of the total number of alleles in a 15-allele and a 32-allele system was estimated (Table 7).

Allele frequencies from 50.1% to 100% are designated high frequency, from 5.1% to 50% medium, from 1.1% to 5% low, and below 1% rare. In a 128-seed family, a frequency below 1% indicates that the allele was observed a maximum of two times in the family. The 15-allele system included four medium frequency alleles (30.95%, 26.59%, 17.86%, and 6.75%), six low, and five rare alleles. The 32-allele system included two medium frequency alleles (39.06% and 27.34%), 12 low, and 18 rare alleles.

Temporal comparison of seed collections

Seeds were collected from four green ash trees and three black ash trees in a high seed production year and again in a low seed production year. The Shannon index was calculated for each family for each year (Table 8).

A Student's t-test showed no significant difference in the Shannon index between collection years. Within this subset of green ash families collected twice, six alleles were unique to the high seed production year while 12 alleles were unique to the low seed production year. Within the black ash families collected twice, 13 alleles were unique to the high seed production year while 8 alleles were unique to the low seed production year.

Discussion

Microsatellite markers

DNA sequences and gene arrangements are highly conserved among closely related species. Because of this it is possible to use primers developed in one species to amplify microsatellites in related species or even other genera within the same family (Kalia et al. 2011). This possibility was evaluated in this study resulting in nine nuclear microsatellite markers developed in *F. excelsior* that successfully amplified black and/or green ash (Table 2). Examples in other genera include primers developed in an olive cultivar (*Olea europaea* L.) that successfully amplified 15 additional *Olea* species (Rallo et al. 2003) and most of the primers developed in European chestnut (*Castanea sativa* Mill.) and Japanese chestnut (*C. crenata* Sieb & Zucc.) that amplified three additional chestnut species (Wang et al. 2008).

However, cross-species amplification does not always work. In this study 15 *Fraxinus* markers were unsuccessful in amplifying both green and black ash and six markers worked in only one species (Table A1). Likewise, primers developed for microsatellites in Eastern white pine (*Pinus strobus*) and Monterey pine (*P. radiata*) had limited success in nine other *Pinus* species and no amplification in white spruce, *Picea glauca* or Douglas fir, (*Pseudotsuga menziesii)* (Echt et al. 1999).

In this study the overall success rate of cross-species marker amplification was 20.83% in black ash and 29.17% in green ash. If enough DNA sequence flanking the microsatellite repeats is known, new primers can be redesigned and tested. Here, primer redesign was successful for FEMSATL11 and for FEMSATL4 in black ash but the known sequence flanking the remaining markers was not sufficient to allow primer redesign.

Most microsatellite repeats are found in non-coding DNA, either between genes or in introns. These are often assumed to evolve neutrally, that is without selective forces acting (Ellegren 2004). The FEMSATL markers and M2-30a were isolated from genomic DNA, the majority of which is non-coding in eukaryotes (Beebee and Rowe 2008) so it is highly likely they came from non-coding DNA. Markers 1502, 7867, 35207, and 43402 were found in a cDNA library made from multiple ash species (Bai et al. 2011), thus they represent transcribed DNA sequence. The sequences containing these microsatellites have not been characterized yet but if they are within or near genes, the potential exists for using the markers to assess functional diversity. A potential consequence of using markers derived from genes is that there is a chance of bias due to selection (Varshney et al. 2005).

Null alleles

Null alleles fail to amplify in PCR because of DNA sequence differences at the site where the primers bind. The presence of null alleles leads to biased allele frequencies such as overestimation of visible alleles and underestimation of observed heterozygosity (DeWoody et al. 2006). It is recommended to discard or redesign primers for a marker showing evidence of null alleles (DeWoody et al. 2006, Selkoe and Toonen 2006). In this study the null alleles were not detected until very late so primer redesign was not attempted. A genotyping system which provided a code for null alleles was devised to work with them. Benefits are that the markers were retained and genotypes were successfully produced in the remaining families. A drawback was that the numbers of seeds used for genetic diversity analysis and inheritance analysis were reduced by 21% to 69% in the families with nulls in the maternal parent (Table 3a, 3b, 5a, 5b). Also, because null alleles were detected in black ash with marker FEMSATL4 and in green ash with markers FEMSATL16 and M2-30a, it is possible that null alleles exist in other families but went undetected when they were not part of the maternal genotype.

Genetic diversity in Fraxinus

This study provides the first estimate of genetic diversity in black and green ash families in Minnesota (Table 3a, 3b). All of the microsatellite markers used here were tested by their developers in various *Fraxinus* species but published studies presenting genetic diversity data in black and green ash were not found. The majority of *Fraxinus* studies using microsatellite markers focus on the European species common ash, *F. excelsior* L. (Heuertz et al. 2004a, Bacles et al. 2005, Ballian et al. 2008)) and to a lesser extent the Asian species Manchurian ash, *F. mandshurica* (Hu et al. 2008, Hu et al. 2010) and the hybridization that occurs between *F. excelsior L.* and narrow-leafed ash (*F. angustifolia*, (Raquin et al. 2002, Fernandez-Manjarres et al. 2006).

Measures of genetic diversity used here to describe families include heterozygosity and number of observed alleles. Observed heterozygosity (H_O) is the mean proportion of individuals that are heterozygous for a set of loci (Beebee and Rowe 2008). It has a maximum value of 1.0 and is proportional to the amount of genetic variability present in the sample (McDonald 2008). Expected heterozygosity (H_E) is the proportion of heterozygotes expected from actual allele frequencies assuming the population is in Hardy-Weinberg equilibrium. H_O and H_E are reported together in this study because discrepancies between them can be clues for the occurrence of null alleles (Beebee and Rowe 2008). Also, family mean H_O, family mean H_E, marker mean H_O, and marker mean H_E, are reported as a means to make quantitative comparisons among the families and among the markers. Global H_O is reported as a means to compare the species. Population genetic measures such as inbreeding, migration rates, test of Hardy-Weinberg equilibrium, estimation of effective population size, and F-statistics were not used here because the samples included in this study are not considered populations.

How do black and green ash compare in terms of genetic diversity? The best comparison can be made with data from the three markers used here in both species. In both FEMSATL11 and FEMSATL19, green ash displayed a larger number of observed alleles (A_O), a wider allele size range, and a higher global H_O (Table 2, 4). In M2-30a, the situation changes and black ash has a larger A_O, a wider allele size range, and a global H_O that is near but slightly below that of green ash. These findings suggest that the black ash families investigated here may be less genetically diverse than the green ash families. This idea is supported by phylogenetic analysis of 40 *Fraxinus* species which indicated that black ash evolved more recently than green ash (Wallander 2008). This history could explain why black ash in some cases has a narrower allele size range than green ash because the mutational processes which create new alleles has had less time to act.

A comparison can be made, albeit cautiously, with *F. excelsior* (Sutherland et al. 2010) using global observed heterozygosity (H_O). Their results place *F. excelsior* global H_O above that of black and green ash for FEMSATL19, between black and green ash for FEMSATL11, and below black and green ash for M2-30a. However, major differences exist in the experimental design between studies. Sutherland et al. (2010) also genotyped seeds, but only one seed per tree was used and the number of seeds genotyped for each marker ranged from 12 to 52. Global H_O was calculated in this study using N=1239 black ash seeds from nine trees and N=1233 green ash seeds from eight trees. Given this information, one cannot say whether *F. excelsior* is more or less genetically diverse than black or green ash. Other *Fraxinus* microsatellite studies vary widely in the type (seeds or trees) and number of individuals tested, along with the number and distribution of populations sampled so it is difficult to compare them objectively (Lefort et al. 2000, Heuertz et al. 2001, Gerard et al. 2006).

It was reported decades ago that green ash is a diploid species with the number of chromosomes 2n=46 (Wright 1959, Black-Schaefer 1989). Most other ash species are also 2n=46 (Koch et al. 2010) but white ash is one of the exceptions with 2n=46, 92, or 138 chromosomes (Schlesinger 1990). Before conducting this study, no specific information was found on black ash ploidy level and chromosome number. Until this data was visualized it was unknown how many alleles to expect per individual. Ultimately, the maximum number of alleles observed per individual was two which strongly suggests that black ash, based on those included here, is diploid.

Mendelian inheritance

Mendelian inheritance of alleles is an assumption for most population genetic analyses. Therefore, it is important to test for it when using a microsatellite marker in a species for the first time. The best way to verify Mendelian inheritance is to perform defined crosses (Selkoe and Toonen 2006). Although it was not possible for this study, controlled crossing has been done in common ash (Morand et al. 2002), silvertop ash (*Eucalyptus sieberi* L. Johnson, not an ash at all) (Glaubitz et al. 2001), and white spruce (Rajora et al. 2001). If defined crosses are not possible, other studies simply indicate whether offspring display at least one maternal allele (Guidugli et al. 2010) or that maternal alleles are evenly divided among offspring (Dow et al. 1995).

Two problems were encountered in this study when investigating inheritance using a chi-square goodness of fit test. First, the chi-square statistic could not be calculated for the families containing a homozygote maternal parent. This is because with only one allele in the genotype, there was only one category in the goodness of fit test, leading to an undefined chi-square statistic for that family. Second, for heterozygous seeds with the same genotype as the maternal parent, it was not possible to determine which of the maternal alleles were contributed and that seed's genotype was not included in the chi square analysis. These seed genotypes were placed in the ambiguous category (Table 5a, 5b). Respectively, these problems led to a reduced number of families tested for each marker and a reduced number of seeds per family.

Mendelian inheritance is suggested in black and green ash for all markers used here (Table 5a, 5b). All markers have at least one family for which there is no significant difference ($p>0.05$) between observed and expected frequencies of maternal alleles. In addition, all seeds except for three shared at least one allele with the maternal parent.

Chi-square analysis indicated a significant difference ($p<0.05$) between the observed and expected frequencies of maternal alleles for Mendelian inheritance in a number of family/marker combinations (Table 5a, 5b). This occurred in both genomic DNA- and transcribed DNA-derived microsatellite markers and in both black and green ash. This could be explained by a disproportionate amount of one of the maternal alleles landing in the ambiguous category. Another possibility is that the microsatellite marker may be linked to a gene under selection.

Optimal sample size for seed collections

Determination of optimal sample size is a challenge attempted in several studies (Lawrence et al. 1995, Sapra et al. 1998, Miyamoto et al. 2008). Information required to determine optimal seed sample size for a particular species includes the number and frequencies of alleles, number of loci, ploidy level, and mating behavior (Sapra et al. 2003). However, at the beginning of this study the number of useful loci for black and green ash and their associated allele numbers and frequencies were unknown. A family size of 128 seeds was chosen based on convenience of sample processing, cost, and available time. Newly optimized microsatellite markers were run on 128-seed families and the resulting data was used to estimate the optimal sample size per tree when collecting black and green ash.

Resampling of data from families displaying 15 and 32 alleles and moderate to high observed heterozygosities (H_O) was performed after Miyamoto et al. (2008).

Figures 1b and 2b illustrate the number of alleles possible when each number of seeds was sampled. To summarize these results, the mean number of alleles from resampling (Figure 1c, 2c) was combined with a 95% confidence interval (Table 6a, 6b). The number of seeds needed to capture 80%, 85%, 90%, and 95% of the total number of alleles in the family/marker was estimated (Table 7).

Given the variation in number of alleles observed when resampling at each level of *N* (Figure 1b, 2b), it made sense to compute a confidence interval to estimate optimal sample size. The 95% confidence intervals are very narrow (on the order of a fraction of an allele, see Tables 6a and 6b) due to the high number of resample iterations (1000) taken at each level of *N*. This means that in families with moderate to high H_O and with 15 or 32 alleles, there is high confidence in capturing the desired amount of genetic diversity when following recommendations in Table 7. Specifically, there is high confidence in capturing at least 95% of the genetic variation identified in a highly variable family with as few as 114 seeds.

This study used collections with a maximum size of 128 seeds per family. However, in the 15 and especially the 32 allele systems examined here, it is likely that this collection size was not sufficient to capture all of the alleles present in these families. This is evident because the slopes of the allelic richness curves for both systems (Figure 1a, 2a) are not at zero as *N* reaches the maximum level.

The 15-allele system consisted of four medium frequency alleles (between 5.1% and 50%), six low (between 1.1% and 5%), and five rare alleles (below 1%). The 32-allele system consisted of two medium frequency alleles, 12 low, and 18 rare. High and medium frequency alleles (those above 5% frequency) represent the current genetic potential while low frequency and rare alleles may represent latent genetic potential, which is important for future adaptation and survival. On the other hand, they may represent deleterious alleles that have been selected against under current conditions (Rajora and Mosseler 2001). It is likely that in both of these families, the medium frequency as well as most low and some rare alleles are captured within 128 seeds and as additional seeds are collected, additional low frequency and rare alleles accumulate.

A conservative approach would be to follow collection recommendations for the 32 allele system even when the allelic diversity of a family is not known. The number of seeds estimated to capture 95% of the diversity in the highly variable 32 allele system is 114 seeds, which has a volume of less than ¼ cup. It is likely that any seed collection effort could easily obtain more than this amount. Certainly it is prudent to scale up the collection size accordingly to have enough seeds for ecological restoration, additional research, and for a backup seed source.

Temporal comparison of seed collections

The Shannon index was developed as a measure of entropy. It has been widely used as a measure of species diversity (Magurran 1988, Shipley and Reading 2006) and is also used to measure genetic diversity (Barrett et al. 2005, Bashalkhanov et al. 2009, Cunha et al. 2012). It is a non-parametric index (Magurran 1988) that measures relative richness and abundance of alleles when used with genetic data. In this study, Shannon index values were calculated in order to compare the genetic diversity between seed collections made in a high seed production year and again in a low seed production year (Table 8).

No significant differences were detected between collection years based on the Shannon index (Table 8). This suggests that the same amount of genetic diversity will be captured in an equivalent number of seeds in both a high seed production year and a low seed production year. With regard to genetic diversity, collection efforts will be beneficial in any year but the collection of seeds will likely be easier and require fewer resources in a high seed production year.

Unique alleles were found in both collection years for both species. These were alleles that could be found in other families, but within the families collected twice they were found only in one of the collection years. In green ash, six alleles were unique to the collection made in the high seed production year while 12 alleles were unique to the collection made in the low seed production year. In black ash, 13 alleles were unique to the collection made in the high seed production year while eight alleles were unique to the collection made in the low seed production year. The finding of these unique alleles could simply be the result of the analysis of a greater number of seeds, as in increasing the family size from 128 to 256. Alternatively, differences may have existed in the number, distribution, and timing of the paternal parents' contribution to the pollen cloud between years.

Chapter 3: Conclusions, management implications, and future directions

Conclusions

Results of this study include a set of nine nuclear microsatellite markers that have been optimized for use in black and/or green ash (Table 2). They demonstrate Mendelian inheritance in black and green ash and therefore can be used in subsequent population genetic studies. Another set of markers have been found to work poorly or not at all in these species and can be avoided in future genetic studies of black and green ash (Table A1).

Based on the analysis of three microsatellite markers in a small number of black and green ash families, green ash may have slightly more genetic diversity than black ash. Further study using additional genetic markers on additional families would clarify this finding. However, it is difficult to compare the genetic diversity of these two species to other *Fraxinus* species because so many differences exist in the plant tissue, population location, sample number, and sample distribution among studies.

Estimates were made for the number of seeds to sample to capture 80%, 85%, 90%, or 95% of the total amount of genetic diversity found in a moderately variable and a highly variable family and marker combination (Table 7). I estimate that 95% of the total variability can be captured in 114 seeds for the highly variable and 109 seeds for the moderately variable family and marker combination. These estimates show that the majority of the genetic information for a family can be captured in just a handful of seeds. Most of the green and black ash seed collections made in Minnesota consisted of several inches of seed in the bottom of a brown paper grocery bag, which represents many times the recommended amount.

Based on the genetic markers and families studied here, there is no significant difference in the genetic diversity of seeds collected from the same trees in different years (Table 8). Alleles unique to each collection are likely to be found, even in a low seed production year. This suggests that with regard to genetic diversity, collection efforts will be beneficial in any year. However, the collection of seeds likely will be easier and require fewer resources in a high seed production year.

Management Implications

In the laboratory:

Information presented here on microsatellite markers in green and black ash (Table 2) is useful for anyone beginning a genetic diversity study in these species. It includes procedures for isolating DNA from ash seeds or buds, a set of markers that have been tested for Mendelian inheritance, and all of the experimental conditions needed to get started. It is also possible that the experimental conditions optimized in this study could be used in other *Fraxinus* species. Finally, materials and reagents for molecular biology are very expensive and knowing which markers to avoid can save time and money (Table A1).

In the field:

Estimates of the number of seeds that capture the desired amount of genetic diversity can be used both in seed collection for conservation and in planting for restoration (Table 8). It is likely that the high frequency alleles will be captured even in a small seed sample and the more seeds that are collected, the more rare alleles will be collected. These rare alleles may represent the adaptive genetic potential of the species or deleterious alleles that are in the process of being selected against (Rajora and Mosseler 2001).

A conservative approach for collection would be to follow recommendations for the 32 allele system even when the allelic diversity of a family is not known. The volume of 114 ash seeds is less than ¼ cup. It is likely that any seed collection effort could easily obtain more than this amount. Certainly it is prudent to scale up the collection size accordingly when additional seed is needed for replication and for storage for future genetic research and ecological restoration.

There is no significant difference in the amount of genetic diversity captured from the same tree collected in different years. This suggests that seeds can be collected whenever is most convenient and when resources allow it.

Future directions

Seeds that are submitted to USDA seed storage facilities are evaluated for filled seed but it would be useful to do germination trials to estimate viability of seed collections. Viability estimates could then be used as an additional factor in resampling to refine the recommended number of seeds to collect from each tree.

It would be valuable to use the nuclear microsatellite markers optimized here to characterize several adult trees in a population and several populations across the state to estimate the within- and between-population genetic diversity found in the species. This information could be used to determine the optimal number of trees per population and the optimal number of populations to visit for seed collection.

Table 1. Maternal parent trees located in Minnesota. Trees for which seeds were collected in both a high seed production year and a low seed production year are indicated by *.

Tree ID	**Species**	**Number of seeds in family**	**City**
MNGA1092	green ash	31	Andree
MNGA1093	green ash	31	Andree
MNGA1095	green ash	31	Andree
MNGA0344	green ash	128	Lynd
MNGA0027 *	green ash	128	Bovey
MNGA0147 *	green ash	128	Cohasset
MNGA0143 *	green ash	128	Deer River
MNGA0365 *	green ash	128	Russell
JDZBA1-1	black ash	31	Audubon
JDZBA2-1	black ash	31	Audubon
JDZBA2-2	black ash	31	Audubon
MNBA0014	black ash	128	Bovey
MNBA0023	black ash	128	Trout Lake
MNBA0115	black ash	128	Big Falls
MNBA0144 *	black ash	128	Bovey
MNBA0007 *	black ash	128	Bovey
MNBA0001 *	black ash	128	Bovey

Table 2. Marker and primer information. BA indicates black ash and GA indicates green ash. Primers that were redesigned from the original source are indicated by *. T_m is the annealing temperature of the PCR cycling parameters.

Marker	Repeat motif	Species	F primer sequence	R primer sequence	T_m	Number of observed alleles	Allele size range	Source
7867	$(TTA)_9$	BA	gaggagataatcggttgcca	gcaatgtcgcttcatattacaa	59°C, 53°C	7	255-276	Bai et. al, 2011
FEMSATL 4	$(CA)_2(AG)_{24}$	BA	tgcttctccgtgtctcagaa*	gctgtttcaggcgtaatgtg	52°C	42	162-248	Lefort et al., 1999
FEMSATL 11	$(GA)_{20}(TA)_4$	BA, GA	ttctttcgcagtttgatagcac*	tggtccagactccagaatgc*	52°C	BA:2 GA:42	BA: 146-148 GA: 146-231	Lefort et al., 1999
FEMSATL 19	$(CA)_6CGGC(CA)_{13}$	BA, GA	ctgttcaatcaaagatctca	tgctcgcatatgtgcagata	52°C	BA:4 GA:10	BA: 119-139 GA: 137-183	Lefort et al., 1999
M2-30a	$(TG)_{15}(AG)_{23}$	BA, GA	cgcacgttctttctatttg	gaccggctgactattttctc	56°C	BA:35 GA:28	BA: 188-279 GA: 183-236	Brachet et al., 1999
FEMSATL 16	$(CA)_3(CG)_1(CA)_{10}(TA)_2(CA)_3$	GA	tttaacagttaactcccttc	caacatacagctactaatca	52°C	14	169-199	Lefort et al., 1999
1502	$(AT)_{10}$	GA	aagcggttaagtaaatgaccca	cggcacaaaaccaaaacag	59°C, 53°C	12	150-168	Bai et. al, 2011
35207	$(ATT)_7$	GA	tttggattcatccatagcca	aagcagaacccagatcaaga	59°C, 53°C	5	253-265	Bai et. al, 2011
43402	$(CCG)_7$	GA	tacccaactttacgcgcact	ttgaaattccccaaaacgag	59°C, 53°C	10	221-254	Bai et. al, 2011

Table 3a. Descriptive statistics in black ash. Large 128-seed families are shaded gray. *N* is the number of seeds successfully genotyped; A_O is the observed number of alleles; H_O is the observed heterozygosity; H_E is the expected heterozygosity; * indicates the known presence of null alleles.

	Family										
Locus	JDZBA1-1	JDZBA2-1	JDZBA2-2	MNBA0001	MNBA0007	MNBA0014	MNBA0023	MNBA0115	MNBA0144	**total**	**marker mean**
FEMSATL 4											
N	28	22	25	127	127	81	101	128	127	766	
A_O	12	14	15	25	23	22	29	32	30	42	26.8
H_O	0.893	*1.000	*1.000	0.906	0.803	*1.000	*1.000	0.734	0.795		0.903
H_E	0.805	0.851	0.850	0.824	0.783	0.854	0.861	0.769	0.801		0.822
FEMSATL 11											
N	31	31	30	127	127	125	126	127	127	851	
A_O	2	2	2	2	2	2	2	2	2	2	2.0
H_O	0.194	0.258	0.367	0.449	0.346	0.512	0.571	0.535	0.496		0.414
H_E	0.178	0.228	0.508	0.349	0.288	0.502	0.410	0.501	0.374		0.371
FEMSATL 19											
N	31	31	31	126	126	127	127	126	125	850	
A_O	2	2	4	3	2	3	3	3	2	4	2.7
H_O	0.194	0.097	0.323	0.984	0.484	0.575	0.283	0.270	0.440		0.405
H_E	0.178	0.094	0.284	0.635	0.438	0.477	0.245	0.235	0.484		0.341
M2-30a											
N	31	31	30	126	125	126	127	125	127	848	
A_O	9	9	12	17	23	15	21	19	28	35	20.5
H_O	0.903	0.710	0.933	0.802	0.800	0.952	0.961	0.832	0.929		0.869
H_E	0.802	0.731	0.812	0.769	0.730	0.795	0.710	0.771	0.796		0.769

Table 3a. Descriptive statistics in black ash, continued

Locus	**Family** JDZBA1-1	JDZBA2-1	JDZBA2-2	MNBA0001	MNBA0007	MNBA0014	MNBA0023	MNBA0115	MNBA0144	**total**	**marker mean**
7867											
N	29	30	30	126	125	125	126	128	127	846	
A_O	4	2	3	4	3	4	3	4	5	6	3.8
H_O	0.483	0.633	0.767	0.270	0.632	0.520	0.516	0.383	0.512		0.524
H_E	0.388	0.440	0.656	0.241	0.573	0.408	0.479	0.314	0.519		0.446
all loci											
mean A_O	5.8	5.8	7.2	10.2	10.6	9.2	11.6	12.0	13.4		
family mean H_O	0.533	0.540	0.678	0.682	0.613	0.712	0.666	0.551	0.635		
family mean H_E	0.470	0.469	0.622	0.564	0.562	0.607	0.541	0.518	0.595		

Table 3b. Descriptive statistics in green ash. Large 128-seed families are shaded gray. *N* is the number of seeds successfully genotyped; A_O is the observed number of alleles; H_O is the observed heterozygosity; H_E is the expected heterozygosity; * indicates the known presence of null alleles.

	Family									
Locus	MNGA1092	MNGA1093	MNGA1095	MNGA0027	MNGA0143	MNGA0147	MNGA0344	MNGA0365	**total**	**marker mean**
FEMSATL 11										
N	31	31	31	126	127	126	128	124	724	
A_O	16	14	15	21	18	22	25	29	40	23.0
H_O	0.871	0.935	0.935	0.905	0.630	0.770	0.914	0.984		0.868
H_E	0.827	0.846	0.819	0.772	0.629	0.768	0.828	0.854		0.793
FEMSATL 16										
N	28	30	31	125	127	126	128	40	635	
A_O	6	5	7	9	6	9	9	11	12	8.8
H_O	0.500	0.367	0.710	0.704	0.661	0.627	0.484	*1.000		0.579
H_E	0.647	0.546	0.648	0.654	0.581	0.610	0.415	0.843		0.618
FEMSATL 19										
N	31	31	31	126	127	126	128	124	724	
A_O	5	7	4	7	7	7	7	7	8	7.0
H_O	0.839	0.839	0.839	0.675	0.795	0.857	0.844	0.790		0.810
H_E	0.714	0.750	0.612	0.607	0.683	0.726	0.749	0.721		0.695
M2-30a										
N	30	31	31	126	85	126	127	126	682	
A_O	14	10	14	18	17	18	15	20	25	17.6
H_O	0.867	0.710	0.742	0.952	*1.000	0.897	0.772	0.825		0.824
H_E	0.796	0.766	0.751	0.817	0.791	0.810	0.764	0.776		0.784

Table 3b. Descriptive statistics in green ash, continued

Locus	Family: MNGA1092	MNGA1093	MNGA1095	MNGA0027	MNGA0143	MNGA0147	MNGA0344	MNGA0365	**total**	**marker mean**
1502										
N	31	31	31	126	127	126	128	125	725	
A_O	7	5	4	8	8	9	7	10	11	8.4
H_O	0.742	0.677	0.710	0.563	0.724	0.500	0.586	0.576		0.635
H_E	0.711	0.611	0.639	0.551	0.710	0.425	0.470	0.467		0.573
35207										
N	31	31	31	126	126	125	128	125	723	
A_O	3	4	3	3	4	4	3	3	5	3.4
H_O	0.581	0.419	0.516	0.119	0.079	0.184	0.164	0.552		0.327
H_E	0.479	0.349	0.524	0.113	0.077	0.171	0.153	0.486		0.294
43402										
N	31	31	31	126	126	126	128	124	723	
A_O	7	7	5	7	7	8	4	8	10	6.8
H_O	0.645	0.323	0.806	0.579	0.762	0.484	0.758	0.742		0.637
H_E	0.677	0.296	0.701	0.560	0.633	0.413	0.652	0.550		0.560
all loci										
mean A_O	8.3	7.4	7.4	10.4	9.6	11.0	10.0	12.6		
family mean H_O	0.721	0.610	0.751	0.643	0.665	0.617	0.646	0.781		
family mean H_E	0.693	0.595	0.671	0.582	0.586	0.560	0.576	0.671		

Table 4. Global observed heterozygosity (H_O). Global H_O was calculated for each species. In black ash N=1239 and in green ash N=1233.

Marker	**Black ash global H_O**	**Green ash global H_O**
7867	0.497	
FEMSATL4	0.846	
FEMSATL11	0.478	0.859
FEMSATL19	0.535	0.767
M2-30a	0.868	0.884
FEMSATL16		0.651
1502		0.598
35207		0.276
43402		0.652

Table 5a. Chi-square test of Mendelian inheritance in black ash. NA indicates the chi-square goodness of fit test is not applicable because the maternal parent is homozygous. * indicates the presence of one seed that did not share an allele with the maternal parent. Number of unambiguous/ambiguous/total genotypes or the number of total genotypes are indicated.

	Family								
Marker	**JDZBA 1-1**	**JDZBA 2-1**	**JDZBA 2-2**	**MNBA 0014**	**MNBA 0023**	**MNBA 0115**	**MNBA 0144**	**MNBA 0007**	**MNBA 0001**
FEMSATL 4	p=0.297 23/4/28 *	p=0.201 22/9/31	p=0.072 25/6/31	p=0.913 81/47/128	p=0.766 101/27/128	p=0.201 120/8/128	p=0.717 122/5/127	p=0.454 114/13/127	p=0.357 118/8/127 *
7867	NA 29	NA 30	p=0.317 25/5/30	NA 125	p<0.001 63/63/126	NA 128	p=0.393 67/60/127	p<0.001 78/47/125	NA 126
FEMSATL 11	NA 31	NA 31	p=0.818 19/11/30	p=0.899 61/64/125	NA 126	p=0.515 59/68/127	NA 127	NA 127	NA 127
FEMSATL 19	NA 31	NA 31	NA 31	p<0.001 58/69/127	NA 127	NA 126	p=0.004 70/55/125	p<0.001 65/61/126	p=0.590 124/2/126
M2-30a	p=0.854 29/2/31	p=1.000 26/5/31	p=0.178 27/3/30	p=0.397 113/13/126	NA 127	p=0.845 106/19/125	p=0.036 110/16/127 *	p<0.001 106/19/125	p=0.041 116/10/126

Table 5b. Chi-square test of Mendelian inheritance in green ash. NA indicates the chi-square goodness of fit test is not applicable because the maternal parent is homozygous. * indicates the presence of one seed that did not share an allele with the maternal parent. Number of unambiguous/ambiguous/total genotypes or the number of total genotypes are indicated.

	Family							
Marker	**MNGA 1092**	**MNGA 1093**	**MNGA 1095**	**MNGA 0143**	**MNGA 0344**	**MNGA 0365**	**MNGA 0027**	**MNGA 0147**
FEMSATL 11	p=0.715 30/1/31	p=0.590 31/0/31	p=0.095 29/2/31	p=0.659 82/45/127	p=0.717 122/6/128	p=0.317 121/3/124	p=0.050 104/22/126	p=0.052 117/9/126
FEMSATL 19	p=0.700 22/9/31	p=0.578 29/2/31	NA 31	p=0.008 89/38/127	p=0.039 114/14/128	p<0.001 105/19/124	p=0.014 73/53/126	p=0.264 97/29/126
M2-30a	p=0.178 27/3/30	p=0.715 30/1/31	p=0.336 27/4/31	p=0.663 84/42/127*	p=0.924 115/12/127	p=0.635 111/15/126	p=0.321 123/3/126	p=0.141 118/8/126
FEMSATL 16	p=0.836 23/5/28	p=0.394 22/8/30	p=0.180 20/11/31	p<0.001 86/41/127	NA 128	p=0.343 40/87/127	p=0.185 82/43/125	p<0.001 87/39/126
35207	p=0.001 21/10/31	NA 31	p=0.172 18/13/31	NA 126	NA 128	p<0.001 76/49/125	NA 126	NA 125
1502	p=0.549 25/6/31	p=0.808 17/14/31	p=0.022 23/8/31	p=0.340 110/17/127	NA 128	NA 125	p=0.118 69/57/126	NA 126
43402	p=1.000 24/7/31	NA 31	p=0.336 27/4/31	p<0.001 84/42/126	p=0.399 90/38/128	NA 124	p=0.152 70/56/126	NA 126

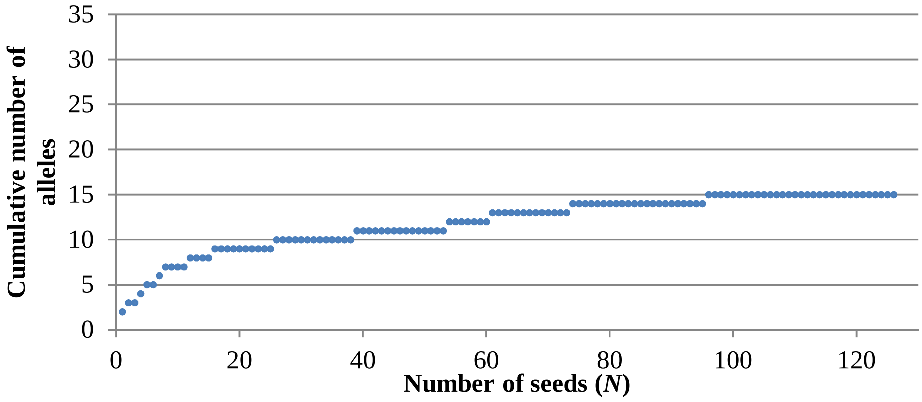

Figure 1a. Cumulative number of alleles detected in *N*=1 to 126 seeds in a moderately variable 15 allele system. Data from family MNBA0014 amplified with marker M2-30a.

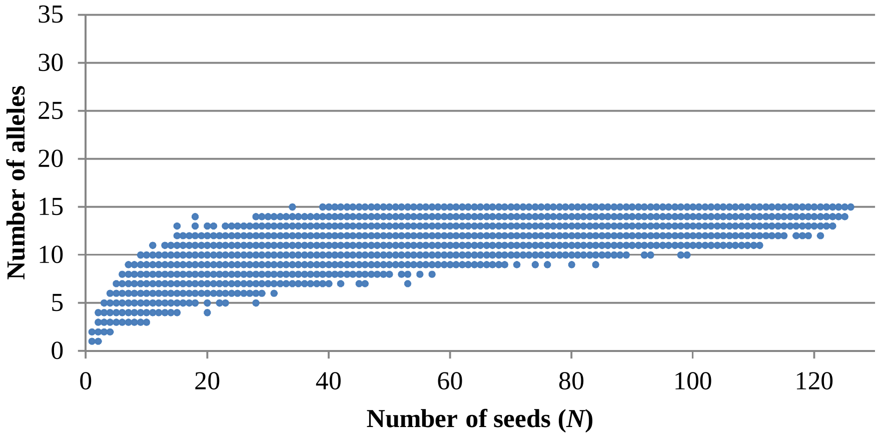

Figure 1b. Number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 126 seeds in a moderately variable 15 allele system. Data from family MNBA0014 amplified with marker M2-30a.

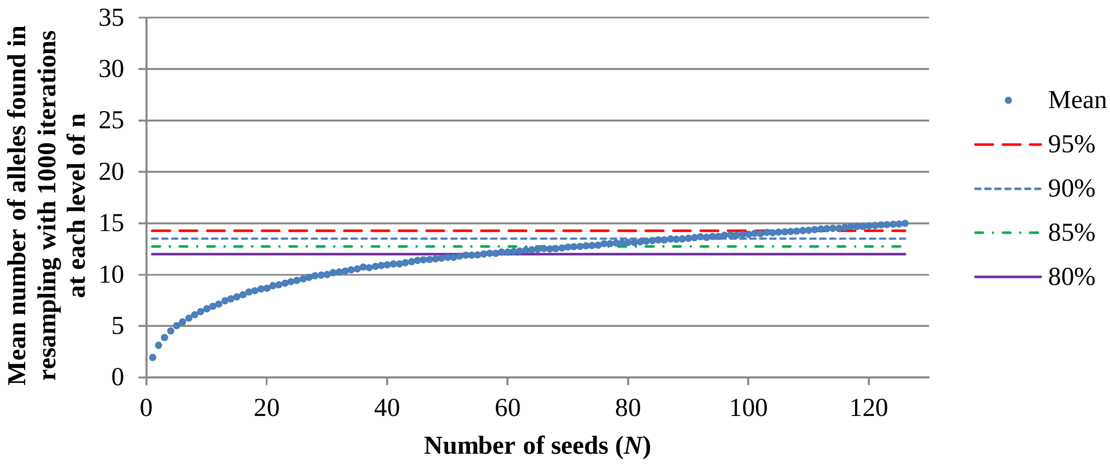

Figure 1c. Mean number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 126 seeds in a moderately variable 15 allele system. Data from family MNBA0014 amplified with marker M2-30a. Horizontal lines indicate the desired level of total genetic diversity.

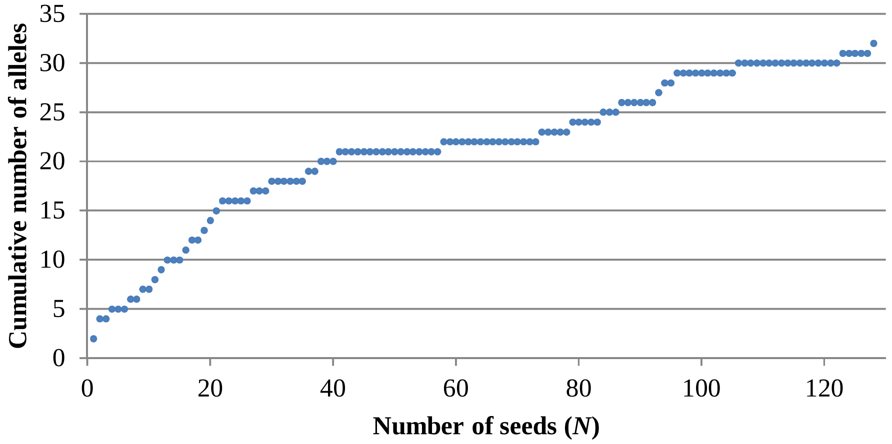

Figure 2a. Cumulative number of alleles detected in *N*=1 to 128 seeds in a highly variable 32 allele system. Data from family MNBA0115 amplified with marker FEMSATL4.

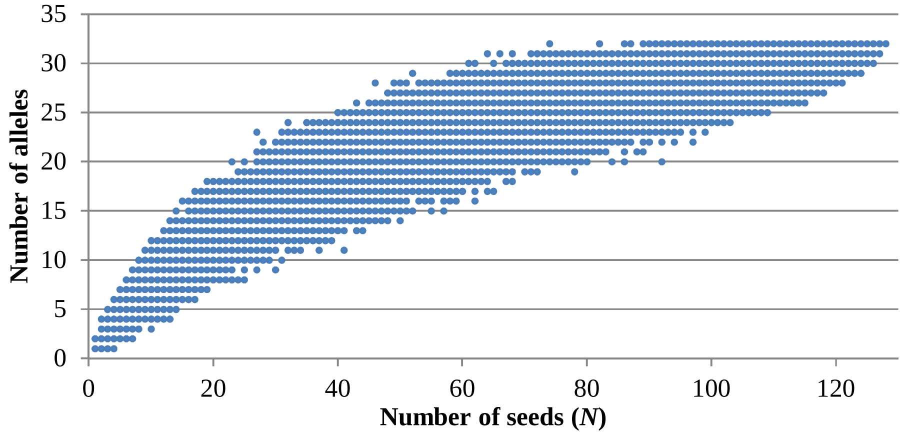

Figure 2b. Number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 128 seeds in a highly variable 32 allele system. Data from family MNBA0115 amplified with marker FEMSATL4.

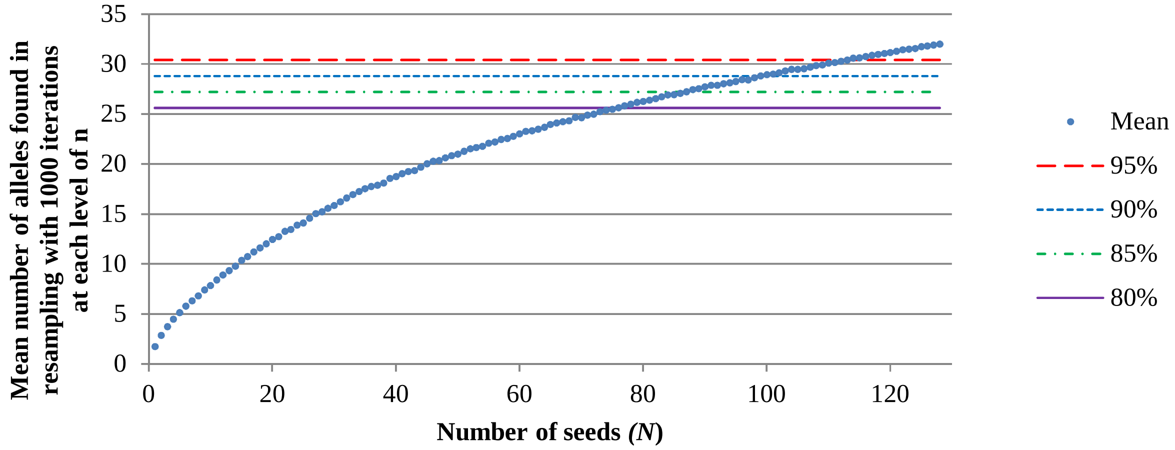

Figure 2c. Mean number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 128 seeds in a highly variable 32 allele system. Data from family MNBA0115 amplified with marker FEMSATL4. Horizontal lines indicate the desired level of total genetic diversity.

Table 6a. 95% confidence interval for the mean number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 126 seeds in a moderately variable 15 allele system. Data from family MNBA0014 amplified with marker M2-30a

N	**Mean # of alleles in 1000 replicates**	**95% confidence interval**	**Bottom of 95% CI**	**Top of 95% CI**
1	1.956	± 0.013	1.943	1.969
5	5.05	± 0.054	4.996	5.104
10	6.69	± 0.071	6.619	6.761
20	8.689	± 0.079	8.610	8.768
30	10.041	± 0.075	9.966	10.116
40	10.985	± 0.075	10.910	11.060
50	11.688	± 0.074	11.614	11.762
60	12.21	± 0.071	12.139	12.281
70	12.694	± 0.073	12.621	12.767
80	13.149	± 0.067	13.082	13.216
90	13.545	± 0.060	13.485	13.605
100	13.957	± 0.055	13.902	14.012
110	14.335	± 0.046	14.289	14.381
120	14.763	± 0.029	14.734	14.792
125	14.959	± 0.012	14.947	14.971

Table 6b. 95% confidence interval for the mean number of alleles detected in 1000 resampling iterations at each level of *N*=1 to 128 seeds in a highly variable 32 allele system. Data from family MNBA0115 amplified with marker FEMSATL4

N	**Mean # of alleles in 1000 replicates**	**95% confidence interval**	**Bottom of 95% CI**	**Top of 95% CI**
1	1.722	± 0.028	1.694	1.750
5	5.133	± 0.061	5.072	5.194
10	7.854	± 0.086	7.768	7.940
20	12.45	± 0.104	12.346	12.554
30	15.834	± 0.119	15.715	15.953
40	18.733	± 0.123	18.610	18.856
50	20.974	± 0.121	20.853	21.095
60	23.009	± 0.123	22.886	23.132
70	24.644	± 0.117	24.527	24.761
80	26.244	± 0.112	26.132	26.356
90	27.706	± 0.101	27.605	27.807
100	28.933	± 0.088	28.845	29.021
110	30.083	± 0.075	30.008	30.158
120	31.136	± 0.052	31.084	31.188
127	31.89	± 0.019	31.871	31.909

Table 7. Recommended minimum number of seeds containing the desired level of genetic diversity in a moderately variable 15 allele and a highly variable 32 allele system. 15 allele system data from family MNBA0014 amplified with marker M2-30a and 32 allele system data from family MNBA0115 amplified with marker FEMSATL4.

		15 allele system	**32 allele system**
desired level of genetic diversity:	80%	57	77
	85%	73	88
	90%	91	100
	95%	109	114

Table 8. Comparison of seeds collected in a high seed production year and a low seed production year. *N* is the number of seeds successfully genotyped; H_O is the observed heterozygosity; H_E is the expected heterozygosity.

	MNGA0027		**MNGA0147**		**MNGA0143**		**MNGA0365**		**MNBA0144**		**MNBA0007**		**MNBA0001**	
Collection date	Sept 2008	Oct 2011	Oct 2009	Oct 2011	Oct 2009	Oct 2011	Oct 2009	Oct 2011	Sept 2008	Oct 2011	Sept 2008	Sept 2011	Sept 2008	Oct 2011
Seed production year	high	low	high	low	high	low	high	low	high	low	high	low	high	low
N	126	127	126	124	127	127	126	125	127	127	127	127	127	127
Total alleles, all markers	73	64	77	87	67	78	88	79	67	58	53	50	51	42
Mean number of alleles per marker	10.4	9.1	11.0	12.4	9.6	11.1	12.6	11.3	13.4	11.6	10.6	10.0	10.2	8.4
Family mean H_O	0.643	0.590	0.617	0.661	0.665	0.704	0.781	0.783	0.635	0.649	0.613	0.639	0.682	0.723
Family mean H_E	0.582	0.562	0.560	0.583	0.586	0.644	0.671	0.669	0.595	0.601	0.562	0.583	0.564	0.573
Shannon index	3.159	3.057	3.244	3.301	3.155	3.390	3.506	3.460	2.880	2.863	2.803	2.828	2.850	2.744
p-value for t-test	0.492		0.744		0.127		0.774		0.912		0.872		0.426	

Bibliography

Anderson, M. K. and G. Nesom. 2006. Black Ash Plant Guide. Contributed By: USDA NRCS National Plant Data Center & the Biota of North America Program.

Applied Biosystems. 2006. Peak Scanner Software version 1.0 Software Reference Guide.

Bacles, C. F. E., J. Burczyk, A. J. Lowe, and R. A. Ennos. 2005. Historical and contemporary mating patterns in remnant populations of the forest tree *Fraxinus excelsior* L. Evolution **59**:979-990.

Bai, X. D., L. Rivera-Vega, P. Mamidala, P. Bonello, D. A. Herms, and O. Mittapalli. 2011. Transcriptomic Signatures of Ash (*Fraxinus* spp.) Phloem. Plos One **6**.

Ballian, D., I. Monteleone, D. Ferrazzini, D. Kajba, and P. Belletti. 2008. Genetic characterization of common ash (*Fraxinus excelsior* L.) populations in Bosnia and Herzegovina. Periodicum Biologorum **110**:323-328.

Barrett, L. G., T. H. He, B. B. Lamont, and S. L. Krauss. 2005. Temporal patterns of genetic variation across a 9-year-old aerial seed bank of the shrub *Banksia hookeriana* (Proteaceae). Molecular Ecology **14**:4169-4179.

Bashalkhanov, S., M. Pandey, and O. P. Rajora. 2009. A simple method for estimating genetic diversity in large populations from finite sample sizes. Bmc Genetics **10**.

Bauer, L. S., R. A. Haack, D. L. Miller, T. R. Petrice, and H. Liu. 2003. Emerald Ash Borer Life Cycle. 2003 Research and Technology Development Meeting.

Beebee, T. and G. Rowe. 2008. An Introduction to Molecular Ecology. 2nd edition. Oxford University Press, New York.

Benedict, M. A. and L. E. Frelich. 2008. Site factors affecting black ash ring growth in northern Minnesota. Forest Ecology and Management **255**:3489-3493.

Benson, D. A., I. Karsch-Mizrachi, K. Clark, D. J. Lipman, J. Ostell, and E. W. Sayers. 2012. GenBank. Nucleic Acids Research **40**:D48-D53.

Black-Schaefer, C., Robert L Beckmann. 1989. Foliar Flavonoids and the Determination of Ploidy and Gender in *Fraxinus americana* and *F. pennsylvanica* (Oleaceae).

Bonner, F.T. and Karrfalt, R.P., eds. 2008. The woody plant seed manual. Agric. Handbk. No. 727. Washington, DC: USDA Forest Service. 1,223 p.

Brachet, S., M. F. Jubier, M. Richard, B. Jung-Muller, and N. Frascaria-Lacoste. 1999. Rapid identification of microsatellite loci using 5 ' anchored PCR in the common ash *Fraxinus excelsior*. Molecular Ecology **8**:160-163.

Cappaert, D. D. G. M., Therese M Poland, Nathan W Seigert. 2005. Emerald Ash Borer in North America: A Research and Regulatory Challenge. American Entomologist **51**(3); 152-165.

Cunha, C. P., E. S. S. Hoogerheide, M. I. Zucchi, M. Monteiro, and J. B. Pinheiro. 2012. New Microsatellite Markers for Garlic, *Allium sativum* (Alliaceae). American Journal of Botany **99**:E17-E19.

DeWoody, J., J. D. Nason, and V. D. Hipkins. 2006. Mitigating scoring errors in microsatellite data from wild populations. Molecular Ecology Notes **6**:951-957.

Dickerson, J. 2002. Green Ash Plant Fact Sheet. USDA NRCS New York State Office, Syracuse, New York.

Dickerson, J. 2006. Black Ash Plant Fact Sheet. USDA NRCS New York State Office, Syracuse, New York.

Dobrowolska, D., S. Hein, A. Oosterbaan, S. Wagner, J. Clark, and J. P. Skovsgaard. 2011. A review of European ash (*Fraxinus excelsior* L.): implications for silviculture. Forestry **84**:133-148.

Dow, B. D., M. V. Ashley, and H. F. Howe. 1995. Characterization of Highly Variable $(GA/CT)_n$ Microsatellites in the Bur Oak, *Quercus macrocarpa*. Theoretical and Applied Genetics **91**:137-141.

Echt, C. S., G. G. Vendramin, C. D. Nelson, and P. Marquardt. 1999. Microsatellite DNA as shared genetic markers among conifer species. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere **29**:365-371.

Eck, David and Jim Ryan. 2012. <u>The Chi Square Statistic</u>. The MathBeans Project. 31 Aug 2012 < http://math.hws.edu/javamath/ryan/ChiSquare.html>

Ellegren, H. 2004. Microsatellites: Simple sequences with complex evolution. Nature Reviews Genetics **5**:435-445.

<u>Emerald Ash Borer</u>. 2012. USDA Forest Service, Michigan State University, Purdue University, Ohio State University. 31 Aug 2012 <www.emeraldashborer.info>

<u>Emerald Ash Borer Prevention, Early Detection, and Rapid Response.</u> 2012. Minnesota Department of Agriculture. 31 Aug 2012 <http://www.mda.state.mn.us/plants/pest management/eab.aspx>

Erdmann, G., Thomas R Crow, Ralph M Peterson Jr, and Curtis D Wilson. 1987. Managing Black Ash in the Lake states. Gen. Tech. Rep. NC-115, St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station. 10p.

Excoffier, L. G. Laval, and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online **1**:47-50.

Fernandez-Manjarres, J. F., P. R. Gerard, J. Dufour, C. Raquin, and N. Frascaria-Lacoste. 2006. Differential patterns of morphological and molecular hybridization between *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl (Oleaceae) in eastern and western France. Molecular Ecology **15**:3245-3257.

Gerard, P. R., J. F. Fernandez-Manjarres, and N. Frascaria-Lacoste. 2006. Temporal cline in a hybrid zone population between *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl. Molecular Ecology **15**:3655-3667.

Glaubitz, J. C., L. C. Emebiri, and G. F. Moran. 2001. Dinucleotide microsatellites from *Eucalyptus sieberi*: inheritance, diversity, and improved scoring of single-base differences. Genome **44**:1041-1045.

Guidugli, M. C., K. A. G. Accoroni, M. A. Mestriner, E. P. B. Contel, C. A. Martinez, and A. L. Alzate-Marin. 2010. Genetic characterization of 12 heterologous microsatellite markers for the giant tropical tree *Cariniana legalis* (Lecythidaceae). Genetics and Molecular Biology **33**:131-134.

Harbourne, M. E., G. C. Douglas, S. Waldren, and T. R. Hodkinson. 2005. Characterization and primer development for amplification of chloroplast microsatellite regions of *Fraxinus excelsior*. J. Plant Res. **118**:339-341.

Hardin, J. W., D. J. Leopold, and F. M. White. 2001. Harlow & Harrar's Textbook of Dendrology. 9th edition. McGraw-Hill, New York, NY.

Heuertz, M., S. Fineschi, M. Anzidei, R. Pastorelli, D. Salvini, L. Paule, N. Frascaria-Lacoste, O. J. Hardy, X. Vekemans, and G. G. Vendramin. 2004a. Chloroplast DNA variation and postglacial recolonization of common ash (*Fraxinus excelsior* L.) in Europe. Molecular Ecology **13**:3437-3452.

Heuertz, M., J. F. Hausman, O. J. Hardy, G. G. Vendramin, N. Frascaria-Lacoste, and X. Vekemans. 2004b. Nuclear microsatellites reveal contrasting patterns of genetic structure between western and southeastern European populations of the common ash (*Fraxinus excelsior* L.). Evolution **58**:976-988.

Heuertz, M., J. F. Hausman, I. Tsvetkov, N. Frascaria-Lacoste, and X. Vekemans. 2001. Assessment of genetic structure within and among Bulgarian populations of the common ash (*Fraxinus excelsior* L.). Molecular Ecology **10**:1615-1623.

Hu, L. J., K. Uchiyama, Y. Saito, and Y. Ide. 2010. Contrasting patterns of nuclear microsatellite genetic structure of *Fraxinus mandshurica* var. *japonica* between northern and southern populations in Japan. Journal of Biogeography **37**:1131-1143.

Hu, L. J., K. Uchiyama, H. L. Shen, Y. Saito, Y. Tsuda, and Y. Ide. 2008. Nuclear DNA microsatellites reveal genetic variation but a lack of phylogeographical structure in an endangered species, *Fraxinus mandshurica*, across north-east China. Annals of Botany **102**:195-205.

Jacobson, K. 2009. Minnesota's Ash Resource and Markets. MN DNR Forest Products Utilization Program.

Jeandroz, S., N. Frascaria-Lacoste, and J. Bousquet. 1996. Molecular recognition of the closely related *Fraxinus excelsior* and *F. oxyphylla* (Oleaceae) by RAPD markers. Forest Genetics **3**:237-242.

Kalia, R. K., M. K. Rai, S. Kalia, R. Singh, and A. K. Dhawan. 2011. Microsatellite markers: an overview of the recent progress in plants. Euphytica **177**:309-334.

Karrfalt, R. P. 2010. Ash Genetic Conservation Plan. USDA Forest Service, National Seed Laboratory, Dry Branch, GA.

Kennedy, Jr., Harvey E. 1990. Green ash. Silvics of North America: 1. Conifers; 2. Hardwoods. Agriculture Handbook 654. U.S. Department of Agriculture, Forest Service, Washington, DC. vol.2, 877 p.

Koch, J. L., D. W. Carey, M. E. Mason, and M. N. Islam-Faridi. 2010. Overcoming Obstacles to Interspecies Hybridization of Ash.*in* 2010 Proceedings Symposium on Ash in North America.

Lawrence, M. J., D. F. Marshall, and P. Davies. 1995. Genetics of Genetic Conservation .1. Sample-Size When Collecting Germplasm. Euphytica **84**:89-99.

Lefort, F., S. Brachet, N. Frascaria-Lacoste, K. J. Edwards, and G. C. Douglas. 1999. Identification and characterization of microsatellite loci in ash (*Fraxinus excelsior* L.) and their conservation in the olive family (Oleaceae). Molecular Ecology **8**:1088-1090.

Lefort, F., G. C. Douglas, and D. Thompson. 2000. Microsatellite DNA profiling of phenotypically selected clones of Irish oak (*Quercus* spp.) and ash (*Fraxinus excelsior* L.). Silvae Genetica **49**:21-28.

Magurran, A. E. 1988. Ecological Diversity and Its Measurement. Princeton University Press, Princeton, New Jersey.
McDonald, David. 2008. Lecture 4. Population Genetics II. University of Wyoming. 31 Aug 2012 <http://www.uwyo.edu/dbmcd/molmark/lect04/lect4.html>
Miyamoto, N., J. F. Fernandez-Manjarres, M. E. Morand-Prieur, P. Bertolino, and N. Frascaria-Lacoste. 2008. What sampling is needed for reliable estimations of genetic diversity in *Fraxinus excelsior* L. (Oleaceae)? Annals of Forest Science **65**.
Morand, M. E., S. Brachet, P. Rossignol, J. Dufour, and N. Frascaria-Lacoste. 2002. A generalized heterozygote deficiency assessed with microsatellites in French common ash populations. Molecular Ecology **11**:377-385.
Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York, NY.
Oksanen, Jari, F. Guillaume Blanchet, Roeland Kindt, Pierre Legendre, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens and Helene Wagner (2012). vegan: Community Ecology Package. R package version 2.0-4.<http://CRAN.R-project.org/package=vegan>
Park, S. D. E. 2001. Trypanotolerance in West African Cattle and the Population Genetic Effects of Selection [Ph.D. thesis] University of Dublin.
Park, S. D. E. 2008. Microsatellite Toolkit v3.1.1. Animal Genomics Laboratory UCD. <http://animalgenomics.ucd.ie/sdepark/ms-toolkit/>
Poland, T. M. and D. G. McCullough. 2006. Emerald ash borer: Invasion of the urban forest and the threat to North America's ash resource. Journal of Forestry **104**:118-124.
Powell, W., G. C. Machray, and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. Trends in Plant Science **1**:215-222.
Primer3. 2009. SourceForge. 31 Aug 2012 <http://primer3.sourceforge.net/>
Rajora, O. P. and A. Mosseler. 2001. Challenges and opportunities for conservation of forest genetic resources. Euphytica **118**:197-212.
Rajora, O. P., M. H. Rahman, S. Dayanandan, and A. Mosseler. 2001. Isolation, characterization, inheritance and linkage of microsatellite DNA markers in white spruce (*Picea glauca*) and their usefulness in other spruce species. Molecular and General Genetics **264**:871-882.
Rallo, P., I. Tenzer, C. Gessler, L. Baldoni, G. Dorado, and A. Martin. 2003. Transferability of olive microsatellite loci across the genus *Olea*. Theoretical and Applied Genetics **107**:940-946.
Raquin, C., S. Brachet, S. Jeandroz, F. Vedel, and N. Frascaria-Lacoste. 2002. Combined analyses of microsatellite and RAPD markers demonstrate possible hybridisation between *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl. Forest Genetics **9**:111-114.
Rogers, L. L. and A. W. Allen. 1987. Habitat Suitability Index Models: Black Bear, Upper Great Lakes Region. Biological Report.

Ruedinger, M. C. D., J. Glaeser, I. Hebel, and A. Dounavi. 2008. Genetic structures of common ash (*Fraxinus excelsior*) populations in Germany at sites differing in water regimes. Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere **38**:1199-1210.

Sapra, R. L., P. Narain, and S. V. S. Chauhan. 1998. A general model for sample size determination for collecting germplasm. Journal of Biosciences **23**:647-652.

Sapra, R. L., P. Narain, S. V. S. Chauhan, S. K. Lal, and B. B. Singh. 2003. Sample size for collecting germplasms - a polyploid model with mixed mating system. Journal of Biosciences **28**:155-161.

Schlesinger, Richard C. 1990. White ash. Silvics of North America: 1. Conifers; 2. Hardwoods. Agriculture Handbook 654. U.S. Department of Agriculture, Forest Service, Washington, DC. vol.2, 877 p.

<u>Seeds of Success</u>. 2012. Bureau of Land Management, Royal Botanic Gardens, Kew Millennium Seed Bank, and others. 31 Aug 2012 <http://www.nps.gov/plants/sos/>

Selkoe, K. A. and R. J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology Letters **9**:615-629.

Shipley, B. K. and R. P. Reading. 2006. A comparison of herpetofauna and small mammal diversity on black-tailed prairie dog (*Cynomys ludovicianus*) colonies and non-colonized grasslands in Colorado. Journal of Arid Environments **66**:27-41.

Simon, Julian. 2012. <u>Resampling Stats</u>. Resampling Stats, Inc. 31 Aug 2012 <www.resample.com>

Simpson, William T. 1998. Equilibrium moisture content of wood in outdoor locations in the United States and worldwide. Res. Note FPL-RN-0268. Madison, WI: U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 11 p.

Sutherland, B. G., A. Belaj, S. Nier, J. E. Cottrell, S. P. Vaughan, J. Hubert, and K. Russell. 2010. Molecular biodiversity and population structure in common ash (*Fraxinus excelsior* L.) in Britain: implications for conservation. Molecular Ecology **19**:2196-2211.

<u>The NPGS Ash Conservation Project</u>. 2012. USDA Agricultural Research Service. 31 Aug 2012 <http://www.ars.usda.gov/sp2UserFiles/Place/36251200/Ash_Project/HomePage.html#coopText>

<u>The Victim: Trees at Risk</u>. 2012. Minnesota Department of Natural Resources. 31 Aug 2012 <http://www.dnr.state.mn.us/invasives/terrestrialanimals/eab/victim.html>

Thormann, I., M. Dulloo, and J. Engels. 2006. Techniques for ex Situ Plant Conservation. *in* Plant Conservation Genetics. R.J Henry, editor. The Haworth Press, Inc, New York.

Toth, G., Z. Gaspari, and J. Jurka. 2000. Microsatellites in different eukaryotic genomes: Survey and analysis. Genome Research **10**:967-981.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes **4**:535-538.

VanderSchaaf, C. L. 2012. Minnesota's Forest Resources 2011. Minnesota Department of Natural Resources.

Varshney, R. K., A. Graner, and M. E. Sorrells. 2005. Genic microsatellite markers in plants: features and applications. Trends in Biotechnology **23**:48-55.

Wallander, E. 2008. Systematics of *Fraxinus* (Oleaceae) and evolution of dioecy. Plant Systematics and Evolution **273**:25-49.

Wang, Y., M. Kang, and H. W. Huang. 2008. Microsatellite loci transferability in chestnut. Journal of the American Society for Horticultural Science **133**:692-700.

Wright, Jonathan W. 1959. Silvical Characteristics of Green Ash. Station Paper NE-126. Upper Darby, PA: U.S. Department of Agriculture, Forest Service, Northeastern Forest Experiment Station. 18 p.

Wright, Jonathan W. and Rauscher, H. Michael. 1990. Black Ash. Silvics of North America: 1. Conifers; 2. Hardwoods. Agriculture Handbook 654. U.S. Department of Agriculture, Forest Service, Washington, DC. vol.2, 877 p.

Appendix

Table A1. Unused microsatellite markers.

Marker	PCR result in green ash	PCR result in black ash	Source
2429	nonspecific amplification	nonspecific amplification	Bai et. al, 2011
9764	peaks are too complicated to genotype accurately	monomorphic	Bai et. al, 2011
53476	monomorphic	monomorphic	Bai et. al, 2011
1502		no amplification	Bai et. al, 2011
35207		monomorphic	Bai et. al, 2011
43402		poor amplification	Bai et. al, 2011
7867	peaks are too complicated to genotype accurately		Bai et. al, 2011
1.19	mononucleotide repeat that cannot be resolved using capillary gel electrophoresis	mononucleotide repeat that cannot be resolved using capillary gel electrophoresis	Brachet et al., 1999
1.26	no amplification	no amplification	Brachet et al., 1999
3.1	monomorphic	unreliable amplification	Brachet et al., 1999
18N	no amplification	no amplification	Brachet et al., 1999
FEMSATL 1	nonspecific amplification	poor amplification	Lefort et al., 1999
FEMSATL 10	no amplification	poor amplification	Lefort et al., 1999
FEMSATL 14	no amplification	monomorphic	Lefort et al., 1999
FEMSATL 16		poor amplification	Lefort et al., 1999
FEMSATL 18	no amplification	no amplification	Lefort et al., 1999
FEMSATL 2	no amplification	no amplification	Lefort et al., 1999
FEMSATL 20	no amplification	peaks are too complicated to genotype accurately	Lefort et al., 1999
FEMSATL 4	peaks are too complicated to genotype accurately		Lefort et al., 1999
FEMSATL 8	no amplification	no amplification	Lefort et al., 1999
FEMSATL 9	no amplification	no amplification	Lefort et al., 1999