POLLINATION BIOLOGY AND SIMPLE SEQUENCE REPEAT (SSR) GENETIC IDENTIFICATION OF CHESTNUT CULTIVARS AND THEIR PROGENY

By

Carmen Medina-Mora

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology-Doctor of Philosophy

2015

ABSTRACT

POLLINATION BIOLOGY AND SIMPLE SEQUENCE REPEAT (SSR) GENETIC IDENTIFICATION OF CHESTNUT CULTIVARS AND THEIR PROGENY

By

Carmen Medina-Mora

After chestnut blight, caused by the accidental introduction of the pathogenic fungus *Cryphonectria parasitica*, ravaged American chestnut (*Castanea dentata*) populations, other chestnut species attracted more interest because blight resistance was of primary concern. Therefore, the Michigan chestnut population consists of American, Chinese (*C. mollissima*), Japanese (*C. crenata*) and European (*C. sativa*) trees and their hybrids. For orchards, selection of grafted cultivars is crucial for long-term commercial success. Due to the mixture of non-grafted seedling trees, hybrid trees, and grafted cultivars in Michigan orchards and the variability of nut production within orchards the two objectives of my study were to 1) genetically identify commercially important chestnut cultivars currently growing in Michigan using simple sequence repeat (SSR) markers, and 2) improve our understanding of chestnut pollination including reproductive phenology, and nut-set using genetic analysis of parents and offspring.

To genetically identify chestnut cultivars growing in Michigan, a total of 110 samples representing 9 European hybrid cultivars and 2 Chinese cultivars were genotyped. The efficacy of 5 previously determined SSR markers to describe the genetic diversity among 8 chestnut cultivars was evaluated using IDENTITY, POPGENE and CERVUS software. The number of alleles per locus ranged from 10 to 19 alleles with intermediate to high levels of heterozygosity (0.457-0.923). Polymorphic information content (0.693-0.797) and power of discrimination (0.707-0.819) were determined. High levels of genetic diversity were observed in the chestnut population included in this study, where 56 genotypes were defined. The overall

SSR profile of each cultivar consisted of alleles useful for the identification of each cultivar included in this study. Unique alleles were obtained with each SSR locus and useful for the identification of 5 out of the 11 chestnut cultivars (‘Colossal’, ‘Benton Harbor’, ‘Everfresh’, ‘Nevada’, and ‘Okei’). Out of the 5 SSR primer sets used, a combination of two primer sets were always sufficient to identify each cultivar, however, the selection of useful primers requires prior knowledge of the cultivars being differentiated. These SSR primer sets were able to identify the parents of F1 progeny when two cultivars, (‘Benton Harbor’ and ‘Okei’) pollinized a third cultivar (‘Colossal’). The SSR-based identification of individual nuts could only be performed if the SSR alleles of the parental trees involved in the cross were known and partially unique to each chestnut cultivar. The SSR profile resulting from the primer sets (EMCs15 and CsCAT1) was sufficient to identify the paternal parent of each nut recovered from the pollination event.

To better understand chestnut pollination, experimental crosses were conducted during the 2008-2010 growing seasons. In controlled, natural pollination experiments, the interaction between pollen and flowers was monitored. For one cultivar ('Colossal'), female flowers were receptive to pollen as early as 19-June, and as late as 3-August. In controlled pollination experiments performed in mid-Michigan, pollen was made available to flowers at pre-anthesis, anthesis, and post-anthesis. The highest level of nut production occurred when pollen was available at anthesis on 11-July, 6-July, and 3-July for the 2008, 2009, and 2010 growing seasons, respectively. Pollen application at anthesis was important to obtain a high number of nuts, however, anthesis shifted from year to year. Studies of genetic characterization and pollination biology should provide opportunities that will help Michigan growers establish and maintain high quality commercial chestnut plantings by improving cultivar identification as well as issues related to pollen timing and nut-set.

To my daughters, Hannah and Kristina, for their endless love.

To my parents, Carmen and Victor, for teaching me the necessary tools and values to succeed in life.

To my late grandmother, Abuela Marina, for being my guardian angel.

To my mentor and friend, Dr. Dennis W. Fulbright, for not giving up on me.

To my friends, especially Nancy Holwerda, for their words of encouragement.

To The Lord, for providing me with strength during many times of weakness.

ACKNOWLEDGMENTS

The author of this dissertation would like to acknowledge the knowledge and guidance provided by her major professor, Dr. Dennis W. Fulbright, and by her committee members, Dr. Andrew M. Jarosz, Dr. James F. Hancock, and Dr. Kyung-Hwan Han. The author would also like to thank Sara Stadt, Mario Mandujano, Irwin Dones-Gonzales, Emily Sweet, Sarah Chesney, Alex Allegretto, and Joseph Dowd for their incomparable help and technical support. Special thanks are due to Dr. Kristina M. Sefc for her valuable technical support and discussion regarding the use of the parentage analysis software, IDENTITY. The author is very grateful for the funding provided by Northern Nut Growers Association, the MSU-Rogers Reserve Endowment, and private funding by Dr. Vladimir Ferrer. Furthermore, the author deeply appreciates the cooperation of various Michigan chestnut growers, especially Mrs.Virginia (Ginger) Rinkel, Mr. Robert (Bob) Rinkel, Mr. William (Bill) Nash, and the late Mr. Kenneth (Ken) LaFever, for allowing the use of their chestnut orchards for experimentation purposes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO SYMBOLS OR ABBREVIATIONS

SSR: Simple Sequence Repeats

X: cross; representing a genetic cross (breeding)

AFLP: Amplified Fragment Length Polymorphisms

RAPD: Random-Amplified Polymorphic DNA

DNA: Deoxyribonucleic acid

RFLP: Restriction Fragment Length Polymorphisms

EST-SSR: Expressed Sequence Tag SSR markers

SNP: Single Nucleotide Polymorphisms

TSP: Temperature-sensitive PCR

PCR: Polymerase Chain Reaction

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

PD: Power of Discrimination

n: haploid, monoploid, 1 set of chromosomes

2n: diploid, 2 sets of chromosomes

3n: triploid, 3 sets of chromosomes

6-FAM: 6-Carboxyfluorescein, fluorchrome

HEX and NED: trade names of fluorochromes

F1: first filial generation

PIC: Polymorphic Information Content

PE: Paternity Exclusion Probability

PI: Probability of Identity

LITERATURE REVIEW

CHESTNUTS

The cultivation of chestnut trees has been significantly impacted by human activity. Paleobotanical, palynological, and historical records suggest that chestnuts have been cultivated in Japan since 5,500 yr. BP (Li et al, 2007), in central China since 4,600 yr. BP (Nishida 1983), and in Europe since approximately 3,700 yr. BP (Conedera et al, 2004). In Japan, chestnuts were considered a major food staple during the Neolithic period, when humans migrated from fish-rich costal regions to central highlands where fishing was extremely limited (Nishida 1983). In central China, natural populations of chestnut trees have been considered an important source of timber and fruits for the Chinese communities in the area for at least the past 2,000 years (Li et al, 2007). In Europe, the introduction of chestnut trees began during the opulent period of the Roman Empire, and its cultivation started when Greeks imported trees for wood and timber. Unlike in Japan and in central China, chestnut cultivation as a food source originated due to socio-economic pressures (Conedera et al, 2004). Contrary to Japan, China, and Europe, chestnut trees in the United States of America have risen from a much recent introduction that dates back to about 2,500 years ago. Paillet (2004) suggested that chestnut trees in North America, originated from human-induced introductions to New England around the 1700's. Miller et al (1996) stated that historic reports indicated that Native Americans were using its fruits during the pre-Columbian era in North America. Recent interpretations on the distribution of the chestnut species in the Asian, European, and American hemispheres concur that the natural colonization of chestnut trees in these hemispheres resulted from the speciation of the chestnut species after

the colonization of trees in glacial refugia created about 15,000 years ago after the last glacial period occurring in the Pleistocene epoch (Li et al, 2007; Lusini et al, 2014; Mattioni et al, 2013).

Chestnut trees belong to the cup-bearing plant family, *Fagaceae* along with other nut-bearing trees like oak (*Quercus*) and beech (*Fagus*) (Bricher and Bricher, 2000). All chestnuts belong to the genus *Castanea*, where *Castanea* species have been subdivided into three sections based on number of nuts produced within a bur (Anagnostakis, 1999; Miller et al, 1996). The first section, the *Eucastanon* corresponds to all the common *Castanea* species whose trees bear three nuts per bur. The *Eucastanon* chesnut species are: the American (*C. dentata* Borkh.), the Chinese (*C. mollissima* Bl.), the European (*C. sativa* Mill.), the Japanese (*C. crenata* Sieb. & Zucc.), and the Chinese dwarf (*C. seguinii* Dode). The second section, the *Balanocastanon* includes two varieties of the same chinquapin (sometimes spelled chinkapin) species, that produce one nut per bur. The singular *Balanocastanon* species is *C. pumila* (Mill.) with two varieties, *C. pumila* var. *pumila* (Allegheny), and C. *pumila* var. *ozarkensis* (Ozark). The third section, the *Hypocastanon* is also represented by a single species, *C. henryi* (Rehd. &Wils.), which originated in China and bears one nut per bur. Dichotomous keys have been formulated to identify each *Castanea* species based on morphological characters (Anagnostakis, 2010; Graves, 1992; Sisco, 2011; Zander, 2000).

Description of *Eucastanon* species

Castanea dentata— Before the 20th century, the American chestnut tree was a co-dominant species of North America's Appalachian forest. The introduction of *Cryphonectria parasitica*, an invasive fungal pathogen, eliminated natural sexual reproduction of this species and any survivors in the natural forest environment are essentially from asexual root collar

sprouts. Before succumbing to chestnut blight, *C. dentata* was recognized for its timber qualities: rapid and straight growth of its trunk, large truck reaching 30-40 meters in length and 1-1.5 meters in diameter, and rot resistant. The bark of *C. dentata* is reddish-brown in color and smooth when trees are young (Sisco, 2011). In older trees, the bark has large ridges and furrows, and becomes silver-gray color. Stems are smooth and hairless with numerous lenticels, and stipules are sharp and fall off in June. The leaves are 5 to 8 inches long, coarsely serrated, oblong-lanceolated, glandular, pinnately veined, and both sides are hairless. The involucre or bur of the American chestnut consists of long (2-3 cm) and slender spines. Each bur contains up to three small (1/2 to 1 inch in diameter), relatively hairy nuts per bur. The hilum (spot) end shows vascular bundles in a sunburst pattern.

Castanea crenata—The Japanese chestnut is native to Japan and southern Korea. Many varieties of Japanese chestnuts are susceptible to chestnut blight, and Milgroom (1995) suggested that its introduction to the United States as rootstock or scion was the mode of entrance of *C. parasitica* to chestnut forest and stands. The Japanese trees could be classified as small, up to 15 meters, and sometimes shrub-like. Young stems are purple-brown with rounded buds. Leaves are crenate, lanceolate, and acuminate, with almost parallel sides and small teeth around edges. Burs spines are short and thick, and create a dense mat of intertwined spines. Commonly, it produces 2 nuts that are usually lateral inside the bur. Some varieties produce large nuts (up to 5 cm in diameter), and have a large hilum.

Castanea mollissima—The Chinese chestnut is native to China, and its seedlings and hybrids were introduced to Korea over 200 years ago. The Chinese trees are more valuable for fruit production than timber, as its trunk may only reach up to 20 meters, and typically have stunted canopy with numerous large and low branches. Its bark is gray-brown to brown,

furrowed, but without distinguishable patterns. Stems are hairy and olive-brown with large lenticels, and stipules are broad and remain attached to stem until September. Leaves are 5-8 inches long, with a blunt end, oblong, alternated, pinnately veined, and coarsely serrated. Chinese chestnut leaves are shiny green and glossy above, and pale green and hairy beneath. Burs have 1-2 cm long and thick spines, and each bur encloses up to three nuts. Nuts are 3/4 to 2 inches in diameter, hairy at one end, and show a diffuse pattern on the hilum.

Castanea sativa— The European chestnut is native to moist temperate regions in the Mediterranean region and Asia. It is also known as Italian, Spanish or sweet chestnut. Since the Roman Empire, European chestnuts trees have been cultivated and propagated by grafting to produce certain varieties unique to particular regions in Italy and France. Trees have a rapid growth, where the trunks are straight (up to 30 meter in length) and large (>1 meter in diameter). Stems are reddish to brown in color and robust. Leaves are oblong-lanceolate with rounded base, and coarsely serrated with triangular teeth. Young leaves are tomentose beneath changing to light green as its ages, while the top of the leaves remains dark green. The petioles of the leaves tend to be longer than in other *Castanea* species. Burs of the European chestnut are similar to the burs of the American chestnut, which consists of long (2-3 cm) and slender spines. Nuts are as big as the nuts of *C. crenata*, and hybrids of these two species produce nuts that can be rather large in diameter. The number of nuts varies from 1 to 3 nuts per bur. The nut size is most likely due to the relative size of the bur. However, burs of the European chestnuts are the largest among the *Castanea* species. Nuts are pointy and hairy at the stylar end, and vascular bundles resemble a star pattern at the hilum.

Castanea seguinii—This *Castanea* species consists of small trees up to 10 meters in height. All trees are members in the *Eucastanon* section, and all trees are native to China. Due to

its short height, the common name used is Chinese dwarf chestnuts. Leaves are lepidote, and glandular on the upper surface and underside. Stems are thin and hairless. Burs consist of pilose spines and enclose very small nuts up to 12mm wide.

Description of *Balanocastanon* species

Castanea pumila— Trees making up this species were considered a taxonomically diverse group until recently when two varieties were grouped into the *Balanocastanon* section. Named chinkapin or chinquapin, *C. p.* vr. *pumilia* is native to southeast North America and it is commonly known as Allegheny chinquapin. The other species *C.p*. vr. *ozarkensis*, is native to Arkansas, Missouri, and Oklahoma, and it is commonly known as the Ozark chinquapin. Trees are small in height up to 15 meters. In general, both varieties have slender, reddish-brown, hairy stems, and buds are covered with scales. Leaves are elliptic-oblong, dark green and tomentose beneath. Burs are spiny and enclose a single, small, oval-shaped, sweet nut. When burs open, they split in half rather than in a star shape, typical of the trees in the *Eucastanon* section.

Description of *Hypocastanon* species

Castanea henryi—Commonly called the Chinese chinkapin, this tree is native to China. This species grows relatively fast, and the trunks remain slender. The tree can attain a height of 25 meters. Leaves are glabrous, slender, and hairless. Burs are long-acuminate usually containing a single nut per bur.

Definition of Cultivar

Due to the timber and edible food bearing habit of these trees, many selections have been made for hundreds of years. Some of these selections should be considered cultivars. According to Article 2 published by the International Code for Nomenclature for Cultivated Plants, also known as Cultivated Plant Code (Brickell et al, 2009), a cultivar is a group of plants that has

been selected for its desired traits or characters, which makes the group unique and distinguishable from other groups, and that its traits or characters remain stable after propagation.

Asexually propagated plants (*i.e*. clones), propagated parts of the plants or different developmental stages of a plant's life cycle, grafting materials (*i.e*. scion or rootstock), grafting-chimera, and groups of seeds derived from open-pollination events involving the identified cultivar, are all considered the same cultivar. A cultivar's name remains unchanged as long as the defined and desired traits are still identifiable. However, atypical plant material propagated from a previously named cultivar retains its original cultivar name, providing that the abnormal phenotype is not due to the propagation and cultivation techniques used.

One of the challenges that could be encountered with this definition begins when attempting to define a cultivar using genotypes instead of phenotypes (*i.e*. genetic sequence vs. observable traits or characters). As an example, European scientists interested in the identification of *Castanea* species have used the term 'true-to-type' during the genotypic characterization of sweet chestnut cultivars primarily from Europe. Thus, as Frankel and Galun (1977) suggested, the 'true-to-type' character of a cultivar must remain constant by the proper maintenance of its original genotype. Breeders should consider the qualities of a cultivar that are traceable to unique genotypic characters, which, for example, could provide a favorable adaptation to different or diverse environment. They also stated that breeders should routinely assess the genotypes of the cultivars of interest by controlling the pollination and the reproductive mechanisms employed by each cultivar. In cross-pollinated species, as in the case of *Castanea* species, proper maintenance of the cultivars will include the isolation of the trees of

interest from foreign pollen, the maintenance of parental trees, and knowledge of the reproductive efficiency of the cultivars of interest.

Anagnostakis (1999) noted that many of the chestnut cultivars available in this century are the result of controlled or accidental breeding efforts to preserve desirable characteristics of the chestnut trees. Many people from various countries, where chestnuts are commercially grown, have selected and propagated certain trees because of nut size, timber quality, resistance to pathogens such as *Cryphonectria parasitica* (chestnut blight), and *Phytophthora cambivora* (ink disease/root rot), and resistance to insects such as *Dryocosmus kuriphilus* (Asian gall wasp). In Anagnostakis' report (1999), scientists, chestnut growers and breeders could find a list of the "named cultivars" including morphological traits for which these cultivars were selected and their putative pedigree.

Another definition to be considered in this study is the meaning of hybrid. All *Castanea* species are cross compatible and can potentially produce offspring with desirable traits that are inconsistent or absent in the paternal species with which it may hybridize. In the past 100 years, breeders have intentionally crossed *Castanea* species to select for interspecific hybrids that harbor desirable traits such as pathogen resistance, adaptations to sub-optimal environmental conditions, nut size and quality, as well as higher pollination and yield potentials. Effectual hybridization events among *Castanea* species have taken place naturally and intentionally, resulting in a challenge for chestnut experts, breeders and growers to morphologically describe the interspecific hybrids (Miller, 2003).

A Brief History of Chestnut Tree Plantings in Michigan Focusing on Germplasm

Most of the pre-1997 chestnut plantings in Michigan consisted of wild American chestnut or (non-grafted, seedling) Chinese chestnut germplasm promoted by various nut grower groups.

The focus on the establishment of *C. mollissima* (Chinese) trees to Michigan chestnut orchards was due to known resistance in this species to chestnut blight. For the purpose of establishing orchards, Chinese chestnut seed was collected from scattered orchard locations, grown in nurseries and commercially sold by various chestnut promotion groups.

The grafted cultivars 'Sleeping Giant', 'Eaton', 'Orrin', 'Mossbarger' and a few others were traditional Chinese/Asian chestnut cultivars that were commercially available for decades prior to the 1980s; however, these trees were not heavily promoted for orchard establishment. In the 1980s, 'Dunstan Hybrid' was the first "cultivar" advertised, sold and planted in Michigan. However, and this came as a surprise to most growers, 'Dunstan Hybrid' was not a grafted cultivar but was a seedling tree propagated only through seed. 'Dunstan Hybrid' was produced by one nursery, Chestnut Hill in Alachua, Florida. This nursery marketed these seedling trees with this name based on a currently expired patent that described an interbreeding consortium of chestnut-blight resistant, American-like chestnut trees. The patent was based on the description of these trees as American-like, blight resistant chestnut trees. Presumably, a cross was made between a Chinese chestnut tree and an American chestnut tree and the result was a hybrid with American features but included blight resistance of it Chinese parent. To produce the 'Dunstan Hybrid', a group of these trees called a consortium were allowed to intercross and the nuts collected. The resultant seedlings were termed 'Dunstan Hybrid', and these trees were produced at the Chestnut Hill nursery. Phenotypically, 'Dunstan Hybrid' is very similar to Chinese chestnut including the presence of Chinese-style leaf trichomes on the underside of the leaves, leaf shape, persistence of stipules and overall general phenotype (although being a seedling, the phenotype is variable from tree to tree). Other nurseries were licensed to plant and sell 'Dunstan Hybrid' under different names such as 'Bond Orchard Selection', 'Linda', and 'Vigorous' from

Forrest Keeling Nursery, Elsberry, Missouri (www. forestkeeling.com). These are all seedling trees. They are not clonally propagated, and are diverse in nature due to the sexual crosses used to produce them. Perhaps thousands of 'Dunstan Hybrid' trees have been planted in Michigan with varying degrees of survival and success.

The next trees to be promoted in Michigan were 'Carolina', 'Willamette', and 'Revival' which are grafted Chinese chestnut trees from the same nursery that produced 'Dunstan Hybrid'. These trees were also patent protected, and were clonally propagated grafted cultivars. These cultivars followed the introduction of 'Dunstan Hybrid' and were also sold by licensed nurseries throughout the east and Midwest. The purchase prices of these grafted trees were prohibitive to orchard plantings and were meant for backyard plantings for hobbyists. A few trees of these cultivars can still be found in Michigan, but they are not common. They are represented at the Michigan Nut Variety trial at Southwest Michigan Research and Extension Center (SWMREC) in Benton Harbor and presumably known to be planted at a few orchards.

The Auburn University series of Chinese chestnut trees ('Au-Cropper', 'Au-Homestead', and later 'Au-Super') from Joe Morgan's program at Auburn University were grafted cultivars that were included in the SWMREC plots in 1992 and later in Clarksville in 2003. These cultivars in Michigan proved to be disappointing as they were slow to grow and produced poor yields.

As orchard plantings initiated with Chinese chestnut seedlings (unnamed) became more common, cultivars were largely ignored. However, by the early 1990s, the seedling-based orchard trees were becoming problematic, slow to go into nut production, and producing varying amounts of chestnuts representing various nut sizes and quality when they did produce nuts. Each tree was distinct from the other in terms of quantity and quality of nut. Most trees rarely

went into nut production within 10 years. Overall, many complaints were received by extension personnel and researchers at Michigan State University (Fulbright and Mandujano, 1999).

The cultivars known as 'Colossal' and ‘Nevada’ both considered European X Japanese hybrid trees (Anagnostakis, 1999) propagated by Fowler Nursery in Newcastle, California were the first widely planted cultivars in Michigan and the first European X Japanese hybrids planted in Michigan. ‘Colossal’ and ‘Nevada’ were planted in SWMREC in 1992 and by 1995, ‘Colossal’ was the largest producer of chestnuts in the trial. Michigan chestnut growers primarily purchased these two *C. sativa* X *C. crenata* cultivars beginning in 1997 as part of a program sponsored by Michigan State University chestnut research. Today, Fowler Nursery no longer propagates and sells chestnut trees, but it should be noted that most of the chestnuts marketed by the Michigan chestnut grower’s cooperative (Chestnut Growers, Inc.) are ‘Colossal’ nuts due to the vast plantings of ‘Colossal’ and the large yields this cultivar has provided growers in Michigan. ‘Colossal’ is the unofficial leader in chestnut production not only because of the large number of trees planted during the late 1990s and early 2000s but also because of its tremendous yield advantage over any other chestnut cultivar planted in Michigan (Fulbright and Mandujano, 1999). Gold et al (2006), at the University of Missouri, have documented the yield advantage of 'Colossal' trees. The nuts from this cultivar are now quickly being recognized for their favorable characteristics such as flavor (Kelley and Behe, 2002; Warmund, 2011) and size.

Both ‘Colossal’ and ‘Nevada’ were first promoted in the western states where chestnut blight is not a problem and most orchard acreage there consists of ‘Colossal’ and ‘Nevada’ plantings. These cultivars are typical of a type of chestnut known as the French hybrid (*C. sativa* X *C. crenata*), that is, typical of those planted in France. It should be noted that neither ‘Colossal’ or ‘Nevada’ came from France, but were part of a breeding program conducted early in the 20th

century by Felix Gillet in California (Miller, 2003). It is said that the ortets, sometimes also referred as mother trees, are still alive in the foothills of the Serra Mountains near Nevada City, California.

In Michigan, 'Colossal' trees could begin yielding an average of more than 4 pounds of chestnuts per tree for commercial and marketing purposes as early as 4 years after planting (Fulbright and Mandujano, 2001). Its most favorable trait, and hence where its cultivar name was derived is its large nut size. Chestnuts from this cultivar are large ranging from 15 to 30 grams per nut (Bassi and Craddock, 1998; Fulbright, 2011). 'Colossal', like all chestnuts, requires out-crossing for nut production because chestnuts are self-incompatible, thus in a clonal chestnut orchard a pollen donor (*i.e*. pollinizer) must be planted in proximity to 'Colossal' for it to bear nuts. Three cultivars 'Silverleaf', 'Nevada' and a few years later, another cultivar, 'Okei', were propagated and sold by Fowler Nursery for this purpose. 'Silverleaf' failed to grow in Michigan and after a severe winter of 2003, 'Nevada' proved to be too cold sensitive in most growing areas. 'Okei', as a replacement tree for 'Nevada' has not been much better. This proved to be problematic as only a limited number of cultivars were commercially available to pollinize 'Colossal' and most were difficult to grow in Michigan. Cultivars of Chinese chestnut were tested for synchronicity with the 'Colossal' pistilate flowers and some proved to have the same flowering dates as 'Colossal', but in actuality, many growers had planted 'Colossal' among their Chinese seedling trees and the 'Colossal' trees were receiving pollen from these Chinese chestnuts.

'Nevada' was introduced to Michigan growers in 1997 because Fowler Nursery suggested it as the best pollinizer for 'Colossal' trees. This cultivar is also, a European-Japanese hybrid (*C. sativa* X *C. crenata*), yet, unlike 'Colossal', it was a cold sensitive tree. This was a surprise for

Michigan growers as the ortet was supposed to be from the same region as ‘Colossal’, the foothills of the western Serra Mountains in California. When 'Colossal' flowers are efficiently pollinized by 'Nevada', 'Colossal' nuts could be on average 20 grams per nut (Fulbright and Mandujano, 2001). ‘Nevada’ does not produce saleable nuts in Michigan as there are several malformed nuts in a bur. ‘Colossal’ being male sterile could not pollinize ‘Nevada’, and a third cultivar was required in the orchard. In western states, ‘Nevada’ has been a reliable pollinizer for ‘Colossal’ and a good nut producer.

'Okei' is a Japanese-Allegheny chinquapin (*C. crenata* X *C. pumila* var. *pumila*) hybrid known for its synchronous-appropriate source of pollen for ‘Colossal’. This cultivar is thought to be a seedling of 'Silverleaf', a well-known cultivar propagated in California with predominant characteristics of Japanese germplasm (Anagnostakis, 1999). After ‘Nevada’ began to fail in orchards in 2003, Fowler Nursery offered ‘Okei’ as a suggested replacement. It does pollinize ‘Colossal’ but it too has trouble with overwintering in some locations.

'Benton Harbor' was the first grafted Chinese chestnut tree produced by Michigan State University for the newly organized chestnut industry. It was a selected seedling tree planted at SWMREC in Benton Harbor, Michigan. This planting also contained some ‘Dunstan Hybrid’ trees, and this tree could be a selection from the ‘Dunstan Hybrid’ trees planted in 1992. The ‘Benton Harbor’ selection was made at SWMREC and the ‘Benton Harbor’ phenotype appears to be very similar to other Chinese chestnuts except that it is precocious, producing copious pollen and pistillate flowers within a couple years after planting and it is vigorous. It also produces the largest yields of high quality nuts of any Chinese chestnut tree in Michigan (Fulbright and Mandujano, 1999).

After observing the ’Benton Harbor’ ortet (*i.e.* mother tree), under Michigan's harsh winter climate, it was selected from other seedling trees and cultivars at SWMREC for commercial production in Michigan. Clonally propagated trees, also known as ramets, from the mother tree were simultaneously produced and planted at other experiment stations in Michigan (Clarksville and Traverse City), provided to growers and planted at the chestnut cultivar repository in New Franklin, Missouri. Besides being the best yielding Chinese chestnut cultivar in Michigan, it also appeared to be able to serve as a good pollinizer of 'Colossal'.

In Michigan, 'Benton Harbor' pollen grains dehisce in early July, when 'Colossal' flowers appear to be receptive. However, its most beneficial characteristic is its vigorous growth, which also makes 'Benton Harbor' vulnerable to wind damage, yet, because of its vigorous growth, 'Benton Harbor' quickly recovers from the damage. Overall, a single tree produces nuts of high quality and a 15 to 20-year-old tree can yield upwards of 70-90 pounds of chestnuts per year, the largest yield of any Chinese chestnut cultivar in Michigan (Fulbright, 2011).

'Labor Day' also known as **'J160'** was discovered as a seedling tree by the late Norman Higgins, Shiawassee County, MI. Others seedling trees planted on his farm included J26, J29, H2, C3, and ‘Labor Day’, were described by Higgins as Korean chestnuts. There is no Korean species of chestnut, however, there are Korean chestnuts thought to be Japanese (*C. crenata*) subspecies. This has not been studied in much detail, however, there are plantings of what have been described as Korean chestnut trees at the USDA laboratory in Beltsville, Maryland (Dr. Fred Hebard, personal communication). Other than ‘Colossal’, the most widely grafted tree in Michigan is ‘Labor Day’. ‘Labor Day’ produces nuts comparable in size to Chinese chestnuts (11 to 14 grams per nut). The ortet is alive and at the farm in Shiawassee County. The most beneficial characteristic of 'Labor Day' is its production of early season pollen and early season

nut production (Fulbright, 2011). This is an important trait for growers with a need for early season production and for those growers in northern climates where late producing chestnuts can experience damaging freezes while still in the bur on the tree.

'Everfresh' is a selection of a Chinese chestnut, similar to 'Benton Harbor'. The 'Everfresh' ortet is an approximately 15-year-old graft on a Chinese chestnut tree also located at SWMREC. Although, 'Everfresh' is a slow growing tree it has favorable traits that promoted its propagation and its distribution to growers interested in the commercialization of chestnuts (Fulbright, 2011). The best trait of 'Everfresh' is its long shelf life. It was determined to have the lowest levels of mycotoxins in studies comparing it with 'Colossal' and 'Eaton' (Donis-Gonzales, 2008). This cultivar consistently produces high quality nuts of medium size (11 to 13 grams per nut).

'Bouche de Betizac' is a French hybrid brought to Michigan because it is thought to be resistant to multiple pests including chestnut blight, *Phytophthora* root rot, and Asian gall wasp (Fulbright, 2011). This *C. sativa* X. *crenata* French hybrid that actually came from France, is well adapted for growing conditions in Michigan. It yields large good tasting chestnuts (15 to 18 grams per nut) in its first years in the orchard, but has a tendency to over produce small nuts as it ages. Its most favorable characteristic among growers is that its pellicle can be easily peeled (Anagnostakis, 1999).

'Precoce Migoule' is also a *C. sativa* X *C. crenata* hybrid well adapted to Michigan's cold climate. It was selected by researchers to be introduced to Michigan because it produces large amounts of male inflorescences or catkins where pollen grains dehisce during 'Colossal' anthesis. This tree has an upright growth habit and its nuts mature approximately two weeks earlier than 'Colossal' (Fulbright, 2011a). This tree may replace both 'Nevada' and 'Okei' as a companion pollinzer to 'Colossal' in Michigan.

Challenges of Identifying Chestnut Cultivars Currently Planted in Michigan

Germplasm is the foundation of good horticulture and planting the proper chestnut germplasm is necessary if a chestnut industry is to become economically sustained in Michigan. However, due to the propensity of growers to plant seedling trees of unknown background and the lack of planting grafted trees in the early years, it is apparent that we do not always know the genetic origins of chestnut trees in Michigan chestnut orchards.

Even now, when most growers are planting grafted trees, keeping track of hybrids and cultivars can be difficult since all *Castanea* species can cross-pollinate. Even in the best horticultural situations, it is possible that cultivars are improperly labeled. There are three common ways for this to occur. First, if grafts fail on grafted cultivars, buds on the rootstock may grow, and unknown rootstock may then be accidentally designated as the cultivar. A second method of mixing cultivars with unknown wild germplasm when amateurs and hobbyists (from whence the chestnut industry arose in Michigan) name seedlings as if they are cultivars, as long as they knew the cultivar mother tree from which the seed arose. A third common mistake occurs when growers, nurserymen and researchers error by mis-labeling trees through various scenarios such as mixing tags or mixing scion wood. The result is that names of cultivars are lost or mixed up.

Knowledge of species and selection of cultivars is an important step when establishing and maintaining orchards because chestnut species and cultivars respond differentially to pests and diseases such Asian chestnut gall wasp and chestnut blight. In two specific cases, one grower stated he grew mostly Korean chestnut, a species not recognized in the taxonomy of chestnut; and in another case, a dwarf chestnut has been identified and we can only speculate as to its

possible pedigree. Beginning a process to help nurseries and growers maintain good genetic records will help establish known germplasm in the new orchards.

Currently, the chestnut industry lacks a certification and validation program of chestnut cultivars. The establishment of genetic markers for chestnut cultivars could help this process (Buck et al 2003; Marinoni et al 2003; Medina-Mora, 1999). Michigan's new chestnut industry could benefit from a reliable method of identification of chestnut species and cultivars. First, as orchards are being planted, growers are dependent on a few national nurseries providing germplasm. Genetic techniques, common today but not currently applied for chestnut, could be used to identify and certify that trees are correctly labeled. Frequently, chestnut rootstock will lose scions or buds after frosts or other damage and the resulting re-growth will be unknown to the grower. DNA analysis would be useful to determine if the tree has grown back from the cultivar or the rootstock. Also, researchers could use such techniques to screen selected trees found on farms that might hold promise in breeding programs or help protect the dissemination of patented or protected germplasm.

MOLECULAR MARKERS

Marker-assisted Breeding

Classical plant breeding has been based on the selection of individuals with desirable phenotypic traits, including a wide variety of characters of interest for the researcher and the general public. A narrow list of the traits historically selected in plants include: high productivity and yield, increase in market value due to color, taste, and increased self-life of product, and resistance to biotic and abiotic stress. After selection, breeders will conduct intensive crosses between desirable parents until advanced-generation lines are achieved. These lines would then be

assessed for the stability of the genes involved with the desirable traits and propagated until the best line is selected as a cultivar. As expected, this process is time-consuming and requires the use of large amounts of land and economic resources. In this genomic era, breeders have taken advantage of the molecular-based information for plants and trees by selecting individuals or populations with desirable genotypic characters in addition to desirable phenotypic traits. Knowledge of the genotypic character(s) of the desired plant(s) will enhance the selection process, because breeders could choose paternal lines based on the genetic relationship among germplasm.

Genetic Markers

Molecular markers have been primarily described as useful tools to study the genetic diversity of crops (Varshney et al. 2005). Moreover, the development of molecular markers are useful in the determination of the genetic quality and population structure of natural forest and orchards, and the quantitative genes of superior trees (Pijut et al. 2007).

Amplified fragment length polymorphisms (**AFLP)**, are generated after the DNA of interest has been digested with restriction enzymes followed by a cycle of PCR amplification. DNA of different fragment lengths (*i.e*. polymorphic bands) are observed after a specific set of primers anneals to complementary sites on the DNA template. Polymorphic bands can be seen if the forward and reverse primers (*i.e*. primer set) anneal at different complementary sites on the DNA templates. Differences in sizes of the DNA fragments are generated after DNA fragments are subjected to enzymatic restriction DNA digest. Ultimately, the variation observed among the DNA fragments are based on the DNA variation inherent within the restriction sites of each DNA fragment (Powell et al. 1996b). AFLP markers are dominant, which is a major drawback for parentage analysis, unless a high number of loci are investigated (Gerber et al. 1999). Powell

et al (1996b) investigated the efficiency of AFLP as a tool to measure genetic relationship among 12 genotypes of soybean. They concluded that AFLP demonstrated the highest level of multiplex ratio but it showed a low level of expected heterozygosity when compared against other markers tested, RAPD, RFLP, and SSR. The marker index, which is based on multiplex ratio and heterozygosity, for the AFLP analysis resulted in a magnitude higher than the other markers tested; however, AFLP lacked the power to discriminate highly similar germplasm lines.

*Enzyme Analyses***: Allozymes** and **isozymes** are variants of the same enzyme with identical function that resulted from different genes due to gene duplication or alleles of the same gene due to point mutations, respectively. The level of variation achieved by isozymes and allozymes as genetic markers is limited because the genes of enzymes can harbor a low level of variation for the enzymes to remain functional. However, there are several advantages of these types of analyses: (1) lower cost when compared to DNA markers, (2) laboratory protocols have been well-defined in "standard" tree species, and (3) that most frequently used enzymes are involved in well-characterized metabolic pathways (Gillet 1999). Villani et al. (1999) reported on the use of allozymes as genetics markers of natural chestnut populations of *Castanea sativa* in Turkey. Pereira-Lorenzo et al (1996 and 2006) characterized a total of 243 Spanish chestnut cultivars based on the use of 12 isozymes. After performing a cluster analysis of the isozyme data, they found that these isozymes were useful to genotype 154 cultivars. Only 89 of the 243 cultivars analyzed belonged in the same cluster group. These data suggest that a high level of synonymy exists among the 89 cultivars, which correspond to accessions collected from two southern Spanish regions. Huang et al (1998) used 18 allozymes to determine the genetic variation among 12 wild populations of American chestnut (*C. dentata*). Scientists concluded that the 12 wild populations of *C. dentata* have an average level of allozyme variation

(approximately 10%) when compared to other species in the genus. This average level of allozyme variation suggested a low level of genetic diversity among the 12 populations of *C. dentata* studied. The unweighted pair group method average (UPGMA) cluster analysis of these data in combination with RAPDs data (see below for description of technique) for the same 12 wild populations of *C. dentata* revealed four groups: the southern populations, south-central and north-central Appalachian populations, and northern populations.

Random-amplified polymorphic DNA **(RAPD)** variation within the primer binding site: This molecular marker is relatively cheap to develop without previous knowledge of sequence data, however its major drawbacks are low number alleles per locus, homoplasy and high level of homology (King et al. 2010). Solar et al (2005) evaluated 244 Slovenian chestnut trees based on phenotypic and genotypic characteristics to determine the genetic diversity within this European region. The researchers used 80 primers to generate RAPD fingerprints for all trees, corresponding to one Mediterranean area and two continental areas. The phenotypic traits evaluated were nut characteristics such as fruit size, color, flavor, and pellicle intrusion. After the comparison of dendrograms created for the pomological traits and the RAPD fingerprints, they concluded that both characters resulted in the same general grouping pattern for all the chestnut trees; six clusters (*i.e*. groups). Four clusters consisted of chestnut trees originating from the continental area; one cluster from the Mediterranean area; and the remaining cluster containing trees from both regions. Together, both analyses indicated that trees from the continental area had the highest level of genetic diversity within the Slovenian-European region.
Restriction fragment length polymorphisms **(RFLP)** can be identified from an individual after large quantities (2-10 micrograms) of DNA are digested with restriction enzymes and blotted onto a hybridization membrane (Southern blot). The variation among individuals is to due to the

specific restriction sites. In the past, radioactive detection methods were employed to determine polymorphisms, however, new non-radioactive labeling techniques have made RFLP, once again, an attractive method of analysis. RFLP is a co-dominant marker, which allows the fast identification of heterozygous individuals in a population. RFLP could be the marker system of choice due to the vast amount of information it can provide regarding the type of polymorphisms (single base pair change, insertion or deletion) among the individuals analyzed. However, RFLP can only be used as a genetic marker if the parental pedigrees of the individuals to be tested are known, and where large quantities of DNA can be extracted. Rafalski and Tingey (1993) indicated that the main disadvantage of the RFLP assay is that it is labor intensive and time consuming, especially in plant genetics and plant breeding where thousand of individuals are usually generated from a single cross and are needed to be screened from a single experiment. Powell et al. (1996b) tested 12 genotypes of soybean and compared the efficiency of several molecular markers including RFLP. In their study, Powell et al (1996b) scored 249 bands out of the 110 RFLP loci previously mapped onto the soybean genetic map. Regardless of the large quantity of bands scored, they found that RFLP analysis provided the lowest effective multiplex ratio and the lowest marker index when compared against RAPD, AFLP, and SSR analyses. However, RFLP was informative when genetic similarities were studied between *Glycine max* hybrids and *G. max* X *G. soja* hybrids. Because of its low hypervariability level, the RFLP assay remains useful in studies of relationships among interspecific hybrids.

Simple Sequence Repeats **(SSR)**, also known as microsatellites, are short repetitive units of less than four nucleotides interspersed throughout eukaryotic genomes. Once a microsatellite region is localized and sequenced within the genome of interest, a 10-30 nucleotides consensus sequence is identified. After a repetitive site is identified, each SSR locus can be categorized into

di-, tri-, or tetra-nucleotides based on the repetitive units of each locus. Once a pair of oligonucleotides complementary to each conserved flanking region upstream and downstream of an SSR locus is designed and synthesized, each pair of oligonucleotides is used as PCR primers, where the forward primer is labeled with a fluorescent dye. Amplicons of different DNA lengths are generated based on the inherent variation in length within the DNA regions of each DNA strand (Powell et al. 1996a). Ultimately, the polymophisms among the DNA strands are observed after each amplicon is resolved using automated-capillary electrophoresis. In the 1990's, a faster methodology was developed where an enrichment technique based on the use of restriction enzymes and cloning yielded a larger number of clones containing a variety of SSR sequences (Edwards et al. 1996). Edwards et al (1996) described a technique applicable to plant and animal species with unknown genome sequence data, which allowed the use SSR-based technology for non-traditional species.

SSR loci become genetic markers when each locus targets a repetitive sequence distributed once in about every 10 kilo base-pairs (kb), and the SSR locus is associated with a phenotypic polymorphism within the individuals of interest. One of the most useful advantages of SSR markers in studies of parentage analysis is the hypervariable character of these co-dominant markers (Gerber et al. 1999). The polymorphic hypervariability observed with SSR markers is due to replication slippage, which corresponds to the mechanism that occurs within the cell nucleus that leads to the formation of allelic diversity (Tautz 1989). The random changes that occur within the repetitive units alter the number of nucleotides within the region, creating amplicons that differ in length, hence the base-pairs number of an SSR-based allele. Since its development, SSR-based technology has been applied to the study of many plant and tree species under many scientific fields including: classification of species, plant conservation, evolution,

geography, phylogeny, pollen flow and seed dispersal, population genetics, and taxonomy. A brief list of plant species, where SSRs have been the genetic marker of choice include: apple (Pereira-Lorenzo et al, 2008), beech (Tanaka et al, 1999), berries (Lewers et al, 2005; Stafne et al, 2005), cacao (Zhang et al, 2009), corn (Rupp et al, 2009), grapes (Cipriani et al, 2010; Thomas and Scott, 1993), magnolia (Isagi et al, 2004), oak (Marsico et al, 2009), peach (Aranzana et al, 2010), pear (Yamamoto et al, 2002), poplar (Tabbener and Cottrell, 2003), pistachio (Hormaza and Wunsch, 2007), rice (Selvaraj et al, 2011), soybean (Tantasawat et al, 2011), sweet potato (Buteler et al, 2002), wheat (Hayden et al, 2008), and willow (King et al, 2010). Although SSRs have been applied to many plant species, the main disadvantage originates when the SSR markers are used to study natural populations. The development of SSR markers and its application in defining genetic diversity in natural populations could be costly, mainly because of the large number of individuals from different genera or species usually tested. SSR genotyping of natural populations can be expensive because the informative value of each SSR marker set needs to be tested against every individual within each population. However various examples have been presented in the literature where a set of SSR markers has been used to genotype individuals of different genera within the same family, as in the case of the *Fagaceae* family (Akkak et al, 2010).

Regardless of these disadvantages, SSRs are the markers most widely used to assess genetic diversity in many species due to its repetitive nature within the genome of each species (Martin et al. 2010b). Gerber et al. (1999) performed a comparison on the parentage analyses of SSR and AFLP markers in a case study of *Quercus petraea* and *Q. robur* mixed population. They concluded that both markers performed equally well during the total paternity exclusion of the oak population, however researchers needed to score 159 AFLP bands to achieve the high

probability obtained with only 6 SSR markers; 0.999. The main drawback reported for AFLP markers is the lack of potential to determine single parent exclusion; which is the analysis required to compare a parent and an offspring without any other information than its AFLP genotype. With only 6 SSR markers, Gerber et al. (1999) were able to determine high levels of paternity exclusion, even when only the genotype of the mother was known.

In a comparative study, Powell et al (1996b) evaluated the efficiency of RAPD, RFLP, AFLP, and SSR as genetic markers in a collection of approximately 500 North American soybean cultivars and accessions. The performance of each marker was evaluated considering three aspects; (1) overall efficiency of distinguishing polymorphisms between two cultivars selected at random from their soybean collection, (2) marker index, which corresponds to the ability of a marker to detect genetic variability, and (3) the ability of marker to infer genetic relationships among cultivars within the collection. The highest level of polymorphism was obtained with SSR and the lowest level when using AFLP and RAPD assays. An intermediate level of polymorphism was obtained with RFLP. The genetic similarity among genotypes was lowest using SSR loci as markers due to the markers high resolving power. As expected, Powell et al (1996b) found that the similarity index was higher within the *Glycine max* subspecies (intraspecies) than between *G. max* and *G. soja* species (interspecies). Using all markers with the exception of RAPDs, they were able to define two clusters within the soybean germplasm; the first cluster consisting of cultivated soybean (*G. max*) and wild soybean (*G. soja*) and the second cluster consisting of only G. max cultivars and accessions. In conclusion, they found that each genetic marker had different properties and usefulness, however SSR assays were the most informative markers based on the demands of plant breeding and population genetics.

Researchers have used SSR markers to perform comparative mapping between genera within the same *Fagaceae* family (Barreneche et al, 2004). Barreneche et al (2004) found that 37 of 47 SSR markers for *Quercus* and 25 of 30 SSR markers for *Castanea* produced amplicons in the non-source species, which increased the number of SSR markers available for use in species with narrow genetic diversity (*i.e*. intraspecies). Results similar to Berreneche et al (2004), regarding the usefulness of SSR markers can be found elsewhere (Akkak et al, 2009; Boccacci et al, 2004; Nishio et al, 2011)

Recently, a variant of SSR technology has been developed and used in studies involving the assessment of genetic diversity within and between populations. These SSR markers, known as genic SSRs or *expressed sequence tag SSR markers* (**EST-SSRs**), possess the same quality, reproducibility and applicability as genomic SSRs, but with less power of discrimination among individuals due to the origin of the repeat within the species genome. The main advantage of the EST-SSR markers over genomic SSRs is its power in studies regarding functional diversity in relationship to adaptation because EST-SSR are markers developed from expressed genomic regions with known or putative functions (Martin et al, 2010b).

Single Nucleotide Polymorphisms (**SNP)** are usually discovered *in silico* from genomic or EST databases, or from direct sequencing data of candidate genes. In comparison to RAPD, RFLP, and SSR, SNP data generate a higher level of genotyping individuals because it consists of single base-pair (bp) differences within a limited region of the genome. Genotyping individuals based on single bp, as in SNP data sets results in simpler analyses; which makes it an easier and quicker data set to be subjected to automation. In diploid species, SNP is biallelic and can be expressed in a binary alphanumeric format. The most attractive quality of SNP genotyping against other methods is its interrogative character, allowing direct comparison of

data collected through time and in different laboratories, even if researchers use different chemistries and techniques. The most prevalent disadvantage of this genotyping method is the use of reliable DNA sequencing data for the species of interest. The genomes of many species of agronomic and economic importance have been sequenced to date and data are available to scientists through open-access genomic databases. In reality, SNP technology is quite challenging when large numbers of individuals need to be analyzed.

In 2009, a variant of SNP genotyping, *temperature-switch PCR* (**TSP**) was developed and tested on cultivated and wild barley by Hayden et al (2009). TSP requires the use of one PCR primer designed for the previously identified SNP DNA region within the genome of the species of interest identified as "allelic-specific primer", and two additional primers named "locus-specific primers" designed to amplify an amplicon not longer than 400 basepairs in length. During amplification, using three PCR primers with two annealing temperatures, single base pair differences among individuals (*i.e*. polymorphism) could be identified at a faster rate than SNP due to the smaller length of the amplicon obtained by TSP. In the case of the identification of homozygotes and heterozygotes, the amplicons for homozygotes result in smaller fragments corresponding to the "allelic-specific primer" when compared against the amplicons resolved for heterozygotes, which will correspond to the "locus-specific primers". Hayden et al (2009) suggested that TSP genotyping could be easily applicable to other plant and animals species including polyploids because it does not require the use of parental genotypes as reference, as is necessary for SNPs- and SSRs-based genotyping.

Concluding Remarks on Molecular Markers

In retrospect, I would have selected TSP-genotyping for my study on DNA-typing *Castanea* cultivars using SSRs (described in detailed on Chapter 2) because it would have been

an easier approach to score polymorphisms among individuals and cultivars, without previous knowledge of parental genotypes and pedigrees of germplasm. In addition, the identification of SNPs would have been easier than in previous years due to the release of the *Castanea* genome. However, the use of SSR and EST-SSR markers remain the most common and useful techniques to assess genetic diversity and parentage (objective described in Chapter 2). Unfortunately, EST-SSR markers for the genus *Castanea* were not available during the preparation of this dissertation, thus EST-SSR genotyping of chestnuts growing in Michigan will not be presented but rather the use of SSR as genotypic identification of chestnuts.

CURRENT USES OF SIMPLE SEQUENCE REPEATS

SSRs as Genetic Markers for Cultivar Identification of Nut-bearing Species

The usefulness of SSRs as genetic markers is evaluated by implementing statistical analyses, which describe the characteristics of each SSR locus to illustrate genetic diversity. Identification of **almond** cultivars: Gouta et al (2010) assessed the genetic diversity of 82 almond genotypes originating from Africa (Tunisia), Europe and America using 10 genomic SSRs, previously identified for *Prunus* species. The goals of their study were to: (1) identify the accessions in the Tunisian National Collection, (2) estimate the level genetic diversity of almond trees in Tunisia, and (3) determine the similarity among the Tunisian, the European, and the American almond cultivars. Gouta et al (2010) were able to unambiguously identify all 82 genotypes because all of the 10 SSR markers used were highly informative and polymorphic. A high power of discrimination was observed with any of the SSR primers used, which ranged from 0.91 to 0.97. They determined that Tunisian almond trees are highly diverse yet distinct from the European and American cultivars. Upon analysis of the UPGMA-based dendrogram,

they were able to define four distinct clusters: cluster A, including 40 Tunisian cultivars; cluster B, including 3 French cultivars, 2 Italian cultivars, 1 Spanish cultivar, and 2 Tunisian cultivars, one of these with unknown Tunisian origin; cluster C, consisting of 8 Tunisian cultivars, 3 American cultivars, 6 French cultivars, 2 Spanish cultivar, and 1 Italian cultivar; and cluster D, including 4 traditional Italian and 4 traditional Spanish cultivars. In conclusion, Gouta et al (2010) were able to determine that Tunisian, although highly diverse, are genetically distinct from European and American cultivars with the exception of the 10 Tunisian cultivars originating from the northern city of Bizerte, Tunisia.

Identification of **hazelnut** cultivars: Boccacci et al (2006) genotyped 78 hazelnut cultivars using 16 SSR loci to determine the genetic relationship among the cultivars, and to determine the parentage of three North American and two French cultivars. Furthermore, Boccacci et al (2006) were interested to verify the existence of synonymies and homonymies among the cultivars. Cultivars considered as synonyms are those cultivars named differently but found to be genetically identical. While, cultivars considered as homonyms are accessions from a single cultivar, named identically but found to be genetically different. After generating UPGMA cluster analysis, researchers noticed that the resulting clusters correlated to the geographical origin of the cultivars. They described four clusters; cluster I corresponding to hazelnut germplasm originating from Italy and Spain, cluster II included hazelnut cultivars from Turkey, and cluster III consisted of cultivars from England and two cultivars with unknown origins. Cluster IV contained cultivars with uncertain origins and pedigrees. Boccacci et al (2006) observed high probability values (above 107 with 95% confidence level) from SSR allele frequencies for the parentage analyses of all North American and French cultivars tested with the

exception of cultivar 'Ennis'. They determined that the putative parents for 'Royal' and 'Fercoril-Corabel' is 'Barcelona' X 'Cosford', even though both cultivars have different geographical origins. They established that 'Barcelona' X 'Daviana' and 'Cosford' X 'Lunga de Spagna' are the putative parents for 'Butler' and 'Imperatrice Eugenie', respectively. As an example of the power of the SSR data, they were able to support the hypothesis of synonymy between 'Gironell', 'Grossal' and 'Grossal de Constanti', where only 'Gironell' was considered the principal hazelnut cultivar in Spain. Boccacci et al (2006) observed an increase in the power of discrimination (PD) when multiple SSR loci were considered to describe the genotype of each individual included in their study. The use of one SSR locus versus four SSR loci resulted in a difference of 42% PD, where the PD of one SSR locus was 50% while the PD of four SSR loci was 92%. Furthermore, the addition of another SSR locus (5 SSR loci in total) resulted in unique genotypes for all 78 hazelnut cultivars, where the PD of these 5 SSR loci was 100%. This work clearly demonstrated the importance of multiple SSR loci to increase the power of discrimination in this method of genotyping.

Identification of **pecan** cultivars: Grauke et al (2003) developed SSR primers to identify 48 pecan and hazelnut accessions representing the *Carya* species germplasm held at the National Clonal Germplasm Repository. Out of the 24 pairs of di- or tri-nucleotide SSR primers evaluated, 19 pairs produced positive amplification for all the accessions tested. Eleven of the 19 pairs revealed polymophisms at both inter- and intra-specific levels. Primer set, PM-CIM20 was the most informative SSR marker because it revealed unique SSR profiles for each of the 48 accessions tested. Among the SSR primers developed, Grauke et al (2003) identified SSR

primers that were species specific or hybrid specific, where positive amplification was observed for either the pecan and hickory species or any of the hybrids tested.

Upon UPGMA cluster analysis, clusters were not observed based on geographical origins for the North American pecan accessions. The fingerprinting potential of the SSR markers was evaluated based on similarity values among the accessions. They found a wide range of similarity values (0.414 – 0.875), where the highest similarity value observed was between 'Brooks' and 'Hirschi' cultivars. In conclusion, Grauke et al (2003) indicated that the use of the pecan/hickory specific SSR primers as fingerprinting tools would require further testing of more closely related samples within each *Carya* species.

Identification of **pistachio** cultivars: Ahmad et al (2003) developed 25 SSR primer pairs to fingerprint 17 pistachio cultivars from Iran, Syria, Turkey and the United States, and 9 commercial samples from Europe and the United States. Of the 25 SSR primers, 14 resulted in positive amplification of all the pistachio nuts or pistachio shells included in their study. Three of the 14 SSR primers targeted two or more SSR loci in the pistachio genome, while the remaining 11 SSR primers targeted one single SSR locus.

In their studies, Ahmad et al (2003) included an UPGMA cluster analysis consisting of only SSR primers pairs with a single SSR locus. Out of the 11 SSR primer pairs, the majority of these revealed polymophisms among all the samples tested. Upon analysis of the UPGMA-based dendrogram, 4 of the 7 Iranian cultivars were grouped in a single cluster, while the remaining 3 Iranian cultivars were grouped into a single cluster with 3 Syrian cultivars. The single American cultivar 'Kerman' and 1 of the 6 Syrian cultivars, 'Jalab', demonstrated unique SSR profiles. These two cultivars, 'Kerman' and 'Jalab', did not cluster with any other of the European cultivars

included in this study, and clustered as individual "outliers" in the UPGMA cluster analysis. All commercial pistachio samples were fingerprinted, but not included in the UPGMA cluster analysis. Ahmad et al (2003) identified 2 Turkish cultivars, 'Siirt a' and 'Siirt b' to be synonymous based on the use of 11 SSR primers, however they indicated the need for more SSR loci to fully establish the synonymy and the "true-to-type" status of the 'Siirt' cultivar.

Identification of **walnut** cultivars: Foroni et al (2007) studied *Juglans regia* cv. 'Sorrento', the most common Italian walnut cultivar using 66 SSR loci. They were able to define the "true-to-type" genotype of the 'Sorrento' cultivar. Using STRUCTURE cluster analysis, they were able to identify two clusters based on the geographic location of the individuals studied. Thus, *J. regia* trees of the cultivar 'Sorrento' should either have the genotype of the Sorrento peninsula or the genotype of Caserta region, otherwise the walnut trees phenotypically similar to 'Sorrento' should be labeled with another name other than 'Sorrento'. Karimi et al (2010) assessed the genetic diversity of Persian walnut (*J. regia* L.) in Iran using 11 previously designed SSR markers for *J. nigra*. All 11 SSR primers tested were polymorphic in *J. nigra*. Twenty-five out of the 63 alleles produced with these SSR primers were present in 5 of the 7 populations tested. Scientists suggested that the low number of alleles (25 out of 63 alleles) present in the majority of the populations tested could be an indication of the selection pressures acting in each population. The level of genetic diversity within each population ranged from 0.614 to 0.709, and generally the diversity within each population increased from north to south. The lowest level of diversity was observed in samples from Tuyserkan (0.614), where the majority of the individual trees belong to the same clonally propagated varieties. The highest level of diversity was observed in samples from Lorestan (0.709), where individual trees resulted from natural hybridization. After

the analysis of UPGMA-based dendrogram, the 7 populations included in this study were grouped into 2 clusters. One cluster consisted entirely of samples from three populations from the provinces of Malayer, Lorestan, and Kurdestan. The other cluster consisted of all the samples from the remaining four populations from the provinces of Hamedan, Tuyserkan, Serkan, and Kermanshan. In the first cluster, Malayer and Lorestan were more closely related, while in the second cluster Tuyserdan and Serkan were more closely related to each other.

Identification of **chestnut** cultivars— Martin et al (2010a) evaluated 7 previously reported SSRs (i.e. CsCAT and EMCs loci; Marinoni et al. 2003 and Buck et al. 2003, respectively) as genetic markers to characterize the identity of 26 European cultivars (n=96) from Italy. Furthermore, Martin et al (2010a) were interested to use SSR markers to determine the presence of synonymy and homonymy. Synonymies are identified when two or more named cultivars have the same SSR profile or genotype, while homonymies are identified when samples of one cultivar have different SSR profiles or genotypes. They found 20 genotypes among the cultivars tested, where all 'Marron-type' cultivars and 6 chestnut-type cultivars from northern and central Italy were represented in Group I and 20 other chestnut cultivars from southern Italy represented Group II on the generated Neighbor-Joining tree. They observed the highest level of heterogeneity with primer CsCAT1 and the lowest with primer EMCs25; where the highest level of similarity was found among the 'Marron-type' cultivars. Of the 'Marron-type' cultivars, 'Chiusa di Pesio' and 'Combai' samples resulted in two unique profiles, while the third profile was shared by 31 samples representing 6 different cultivars. Regardless of the low level of heterogeneity among the 'Marron-type' cultivars, they found four loci (CsCAT2, CsCAT3, CsCAT16 and EMCs38) with two unique alleles at each locus for the remaining samples. They indicated that the use of

only one of these four specific loci provides enough information to identify 17 cultivars because each locus exposed alleles not found in the total number of cultivars used in their study. Three cases of homonyms were established; samples within each of the 'Bracalla', 'Rigiola', and 'Lucente' cultivars had different genetic profiles. Two examples of synonymies were reported; samples corresponding to 'Cardaccio' and 'Perticaccio', and 'Inserta' and 'Curcia Speciale' were in the same groups with the same genetic profiles.

A vast number of reports have been recently published throughout multiple European countries: France, Greece, Italy (Botta et al, 2005; Martin et al, 2010b), Portugal (Costa et al, 2005), Spain (Martin et al, 2009a; Martin et al, 2009b; Martin et al, 2010b), Turkey, Czech Republic (Hozova et al, 2009), and United Kingdom (Buck et al, 2003). Together, these reports demonstrate SSRs as the most common and useful technique for the identification and classification of *C. sativa* cultivars and accessions, and the detection of homonymies and synonymies within European sweet chestnuts. Similar reports have been published for *C. crenata* (Nishio et al, 2011; Tanaka et al, 2005)*, C. henryi, C. mollissima*, and *C. seguinii* cultivars (Aldrich et al, 2003; Inoue et al, 2009; Tanaka et al, 2005; Wang et al, 2008). Han et al (2007) conducted a study to assess the genetic diversity among *C. mollissima*, *C. seguinii*, and *C. henryi* using inter-simple sequence repeats (ISSR), a variant of SSR, which corresponds to the flanking regions between microsatellite repeats. They suggested that one of the *C. mollissima* cultivars used in the study might have been an ancestral species to all three species.

Recently, various scientists interested in genetic differentiation among *Castanea* species have reviewed the ancestry of a vast number of interspecific cultivars. For example, Liu et al (2013) studied 28 natural populations of Chinese chestnut originating in central China. They conducted an assessment of these *C. mollissima* populations using 8 genic SSRs and 6

chloroplast-targeted SSR. Upon STRUCTURE clustering, they found high levels of genetic diversity among the samples included in their study, but no unique alleles per population that will allow them to cluster the populations based on geographical origin. In conclusion, Liu et al (2013) suggested that the only explanation for the high level of diversity was the natural cross-pollination of the species, and that the only geographical pattern that they could observed was consistent with the location of 4 predominant mountains (Dabashn, Wushan, Qinghangfeng, and Daloushan) in central China. Contrary to Liu et al (2013) findings, studies conducted by Nishio et al (2014) demonstrated that 60 natural Japanese chestnut populations distributed in the Tanba region could be divided onto two STRUCTURE clusters. Nishio et al (2014) conducted an assessment of these *C. crenata* populations using 96 genic SSRs and 79 expressed sequence tag (EST)-SSRs. Upon STRUCTURE clustering, they found high levels of genetic diversity among the samples, but the level of diversity did not interfere with STRUCTURE to predict the two distinct groups based on the geographical origin of the samples. All of the cultivars included in the Tanba cluster were representatives of the Tanba region (Kyoto, Osaka, Hyoko Prefectures and Tanbaguri). Nishio et al (2014) concluded that the cultivars in the Tanba region, hence in the Tanba cluster, should be considered the ancestral cultivars of *C. crenata* since these cultivars have been cultivated in Japan for more than 250 years. In Europe, Lusini et al (2014), Martin et al (2012), and Mattioni et al (2013) reported similar findings in regards to level of genetic diversity of *C. sativa* populations in Bulgaria, Spain, and Italy, respectively. Lusini et al (2014) demonstrated that 336 wild chestnut trees from Bulgaria could be grouped into three distinctive STRUCTURE clusters upon describing each trees with 8 SSRs. Martin et al (2012) were able to define three distinct STRUCTURE clusters for 16 European chestnut populations with Spanish origin. Although all 16 *C. sativa* populations included in their study shared a common gene pool,

each cluster represented one geographical region (southeast, northwest, and northeast) in Spain. Mattioni et al (2013) conducted a similar to the study conducted by Martin et al (2012), however, Mattioni et al (2013), included 4 C. sativa populations from Greece, 14 populations from Turkey, 9 populations from Italy, and only 4 populations from Spain. Upon SSR genotyping using 6 SSR markers and STRUCTURE clustering, Mattioni et al (2014) demonstrated the low levels of genetic divergence among all 779 chestnut samples, and described 2 gene pools corresponding to two broad geographical areas, eastern Europe (Greece and Turkey) and western Europe (Spain and Italy).

SSRs as Markers to Assess Genetic Diversity in Chestnuts

SSR markers are the preferred molecular markers for chestnut genomic studies for the same reasons as described above, and due to the ease of automation of large data sets including the availability of software packages to perform necessary statistical analyses. A large number of studies have been presented where SSRs have been used in the identification and validation of European (Gobbin et al, 2007; Martin et al, 2009a; Martin et al, 2009b; Martin et al, 2010a; Martin et al, 2010b; Pereira-Lorenzo et al, 2011), Chinese (Han et al, 2007; Inoue et al, 2009; Wang et al, 2008) and Japanese (Nishio et al, 2014; Tanaka et al, 2005; Yamamoto et al, 2003) chestnut cultivars. For example, Nishio et al (2011) developed a new set of 12 SSR primer pairs using the *C. crenata* genome sequence that was useful to fingerprint six *Castanea* species. A total of 206 chestnut accessions were included in their study, which corresponded to 129 *C. crenata*, 5 cultivars of Korean origin, 38 *C. mollissima*, 28 Japanese-Chinese hybrids, 1 *C. seguinii*, 1 *C. henryi*, 2 *C. sativa* of Italian and French origin, and 9 wild *C. dentata*. Nishio et al (2011) obtained a total 136 alleles at the 12 SSR loci of all the *Castanea* species analyzed. A

high level of heterozygosity (average of 0.65) was observed for all the Japanese cultivars. Upon analysis of UPGMA-based phenogram, researchers were able to group all accessions into 3 clusters, where cluster A contained all the cultivars originating from Japan with the exception of the Japanese-Chinese cultivars; cluster B, consisted of all the cultivars originating from China as well as all 28 Japanese-Chinese hybrids; and cluster C, grouping all accessions of the American chestnut (*C. dentata*) along with the single accession of *C. henryi*. The 2 European cultivars and the single accession of *C. seguinii* represented a small cluster considered as an "outlier" group. All 5 accessions of Korean origin collected in Japan grouped within cluster B along with all the Chinese cultivars and all the Japanese-Chinese hybrids. Furthermore, the scientists were able to identify 21 cultivars considered to be synonymous because they all shared the same unique genetic profile for all 12 SSR loci. In conclusion, Nishio et al (2011) were able to describe genetic diversity and establish the composition of Japanese germplasm, as well as the genetic relatedness of *C. crenata* to the other five *Castanea* species.

As genic databases are becoming available, a “new” series of SSR primers based on functional genes has been developed. This “new” series of SSR primers are collectively known as expressed sequence tag (EST)-SSRs, and are usually used to estimate functional diversity among the strains genotyped even if the strains/samples are taxonomically distinct. For example, McCleary et al (2013) selected 11 EST-SSR markers developed for the characterization of Chinese chestnut species (*C. mollissima*). They selected markers, which sequences are in proximity to genes or within genes (exons) translated during cell differentiation of KNOX genes (meristem maintenance and function), and various transcriptional factors and protein kinases involved with the plants’ response against pathogens and trichomes development. A total of 214 chestnut samples were included in their study, which corresponded to 65 cultivars: 29 cultivars

of *C. mollissima,* 18 Japanese, Chinese, and European hybrids, 2 with conflicting pedigree records, and 2 unknown cultivars. To test homonymy and synonymy, McCleary et al (2013) included samples from 36 cultivars, which trees were clonally propagated. Upon EST-SSR genotyping and grouping samples using STRUCTURE software, they found high levels of homonymy and synonymy among the cultivars in this data set. All cultivars expected to be synonymous, cultivars with identical genotype with different names, were not. For example, in their study they defined STURCTURE group 8 as a group with unexpected synonymies because 6 out of 8 cultivars in the group ('AU Homestead', 'Kohr', 'Willamette', 'Hong Kong', 'Byron', and 'Eaton') had identical EST-SSR genotypes but their pedigrees are different. High levels of homonymy were observed in this data, where 25 out of 36 cultivars with the same name were assigned to different groups by STRUCTURE software. For example, all 7 samples of the Chinese (*C. mollissima*) cultivar 'Hong Kong' belong to STRUCTURE groups 8, 13, and 18. Interestingly, all 6 samples of the European-Japanese cultivar 'Colossal' belong to STRUCTURE group 5, in agreement with its clonal propagation, however, this group included 2 samples of cultivars with Japanese (*C. crenata*) ancestry, 'OK Kwang' and 'Bisalta #3'. Furthermore, all 4 true-to-type French cultivars (*C. crenata* x *C. sativa*) included in this study belong two STURCTURE groups, none of these including 'Colossal'; (1) group 1, only consisting of samples from 'Bouche de Betizac', and (2) group 2, consisting of samples from 4 cultivars, 'Marigoule', 'Maraval', 'Marsol', and 'Precose Migoule'.

SSRs as Gene Markers to Perform Paternity Analysis in Chestnuts

The use of SSR as gene markers have been applied to many forest trees and crops, however a limited number of studies involved the application of paternity analysis using natural

populations. Therefore, it is significant to note the scientific contribution on this topic from a study conducted by Hasegawa et al (2009). They used 11 previously designed SSR primers specific for *Castanea* species to determine the pollen donor composition of a natural population of Japanese chestnut (*C. crenata*). They performed paternity analyses on pollen grains collected during two stages of sexual reproduction; pollination and seed-set. A total of 281 *C. crenata* trees were included in their study, and 3 out of the 281 trees were used as the source of pollen. The remaining 278 trees were fingerprinted with the same 11 SSR markers because all of the 278 trees were considered to be potential pollen donors. A total of 3,571 pollen grains were collected from the stigmatic surface of 70 flowers, and a total of 304 nuts (seeds) were harvested from 118 burs from the three maternal trees. They found the self-pollen rate was significantly higher at pollination stage than at seed-set stage; 90% and 0.3% respectively. However, the genetic relatedness between the paternal and the maternal trees, as well as the frequency distribution of the distance between paternal and maternal trees at pollination and seed-set stages were not significantly different. Furthermore, they found that individuals within 80 meters of each other and in the same plot were more closely related to each other than if the individuals were further apart from each other (more than 80 meters). After genotyping the seeds of each plot, they found that offspring within each plot were more closely related to each other than to offspring between plots. Upon comparison of the genotypes of offspring from each plot, they were not able to reject the presence of biparental inbreeding, which refers to mating between close relatives.

POLLINATION BIOLOGY OF CHESTNUT TREES

Pollination is essential in angiosperms for the fertilization of ovules and the development of fruit. Biotic and abiotic pollination exists in nature; where the former is driven by the feeding

behavior of insects (*i.e*. entomophily), and the latter is dependent on wind (i.e. anemophily) or water movement (*i.e*. hydrophily). Like most of the trees in the *Fagaceae*, chestnut is anemophilous (Akerman, 2000; Hyde and Adams, 1958; Proctor and Yeo, 1973; Soltesz, 1996), and fruit-set is dependent on the morphological traits and the interactions of its inflorescences. Earlier reports suggested the importance of insects, in particular honeybees, as vectors of chestnut pollen (Frankel and Galun, 1977; Meehan, 1879; McKay, 1972; Stanley and Linskens, 1974). However, experts on pollen morphology agree that chestnuts are wind-pollinated trees because of the similarities in size and shape of chestnut pollen grains to other wind-pollinated trees like oak, and the large number of chestnut pollen grains present in the air (Proctor and Yeo, 1973). Independently, Benedek (1996) and Rutter (1990) noted that chestnut pollen grains are scented and produce nectar, which are characteristics of insect-pollinated male flowers, however chestnut female flowers do not have the suitable size to favor entomophily. A limited number of experiments have been conducted to test either theory (De Oliveira et al, 2001; McKay, 1972). It is interesting to note that the majority of the published references indicating entomophily as the pollination mechanism for chestnuts included Asian or European chestnut species (Gaoping et al, 2001; McKay, 1972). De Oliveira et al (2001) conducted pollination experiments in Portugal for two consecutive years where flowers of 6 European chestnut cultivars were covered with either thick layers of muslin allowing self-pollination or thin layers of muslin inhibiting insects to reach flowers. A third group of flowers remained uncovered (control). They observed no significant differences on yield between both treatments with the exception of cultivars 'Judia' and 'Martainha'. Chestnut production was significantly higher when flowers of 'Judia' and 'Martainha' were covered with thick muslin, yet not higher than the uncovered (control). Also, they observed that the highest chestnut production came from control flowers, where the presence of insects led

to a 2.5 fold yield increase. These researchers concluded that insects played an important role on chestnut production and that chestnut is an entomophilous species.

As researchers attempted to provide evidence in support of entomophily, they disregarded the potential negative effect the muslin treatments may have had on yield, resulting in an erroneous conclusion. Regardless of the pollination mechanism employed by chestnuts, researchers agree that climatic events such as rain, wind currents, and temperature directly affect the phenology of the flowers, the efficiency of pollination, and ultimately fruit production (Frankel and Galun, 1977).

All *Castanea* species are monoecius (Clapper, 1954; Percival, 1965), however, cross-pollination seems to be essential for fruit formation (Dinis et al. 2009, Miller et al. 1996; Miller 2003). McKay (1942) conducted controlled pollination experiments to determine the impact of self-, cross- and open-pollination on fruit set. He observed that fruits developed only in about 1% of the flowers pollinized with pollen grains from the same tree. A limited number of reports have been published where self-incompatibility has been rejected as the primary mechanism of pollination (Clapper, 1954; Ohata and Sato; 1961; Solignat, 1958), because most experts on chestnuts agree that chestnuts are wind-pollinated and self-incompatible trees. Clearly, the understanding of fruit production in chestnuts will be enhanced with more knowledge on the processes of pollination, fertilization and ultimately, embryo development.

Pollination Process

Pollination is the process by which pollen grains are conveyed in plants. This process includes aspects of flower anatomy and reproduction, as well as the formation of gametes. In angiosperms, reproduction involves the interaction of two generations; the gametophytic stage and the sporophytic stage. Survival and perpetuation of most plant species is dependent on the

balance between these two stages, known as the alternation of generations. In a diploid (2N) plant species, the sporangia within the sporophyte undergo a series of meiotic divisions; where the genetic material (*i.e*. chromosomes) is reduced from a diploid state to a haploid state. After a series of mitotic divisions, the male and female gametes are formed and packaged into specialized cells; haploid (1N) pollen grains and haploid ovules, respectively. The behavior of the homologous pairs of chromosomes during meiosis, such as independent assortment and crossing over, leads to genetic diversity within the gametes. At the time of chromosomal recombination, chiasmata may occur when homologous pairs of chromosomes exchange fragments of the genetic material (*i.e*. DNA) during crossing-over prior to meiotic telophase.

Dehiscence of pollen grains is the end result of mature anthers while sessile ovules are the outcome of mature ovaries. In general, angiosperms consist of flowering structures where pollen grains are accessible for dispersal and ovules are protected from harsh environmental conditions to guarantee the development and survival of the zygote. The mechanics of the pollination process, either self-pollination or cross-pollination are primarily dependent on the arrangement and location of the anthers and the ovaries in the flowers of each species.

Chestnut Inflorescences

Monoecious chestnut trees produce two different types of flowering branches; (1) unisexual, carrying only staminate or male flowers, and (2) bisexual, consisting of pistillate or female flowers and staminate flowers (Miller, 2003).

Male inflorescences mainly develop in the central region of the shoot representing a cymose; flat-topped flower cluster, yet unisexual catkins can also develop at the base of flowering branches representing a double cymose (Dibuz, 1996). Moreover, male inflorescences can be found surrounding bisexual flowering branches. Staminate flowers are borne on long

spike-like structures, commonly called catkins. Catkins are often pendulous and flexible (Percival, 1965). The length of the catkins varies among species and cultivars, however, on average, catkins are 15 to 20 cm long and 1.5 cm thick. In most *Castanea* species, each flowering shoot contains ten or more male catkins, which mature at different rates through the season. Each catkin consists of a spiral arrangement of male florets or glomerules. In *C. sativa*, each floret is a composite of up to 7 male flowers (Mert and Soylu, 2006). The number of florets per catkin varies among species and genotypes, and it is dependent on the length of individual catkins (Mert and Soylu, 2006). Queijeiro et al (2006) described 9 morphological traits to differentiate 15 cultivars of *C. sativa* growing in Spain. They found that the number of florets vary as much as 56 to 156 florets per catkin even among cultivars of the same *Castanea* species.

European researchers have established guidelines to describe the morphology of a catkin based on length of stamens and pollen fertility (UPOV, 1988). In order of short to long, catkins are classified as: brachystaminate (1-3 mm), mesostaminate (4-5 mm), and longistaminate (7-9 mm); all with fully functional pollen (Bounous et al, 1992b). However, not all chestnut cultivars produce fertile pollen and these are usually classified as male sterile. The catkins of these cultivars are classified as astaminate catkins because of the absence of any of the parts of the androecium (i.e. filaments, anthers, and pollen) within each floret (Bounous et al, 1992a). Mert and Soylu (2006) described fertile and sterile anthers among four cultivars of *C. sativa*. They determined that one floret of *C. sativa* has on average between 70 to 72 stamens. Two levels of development could be described for each catkin: (1) the opening of florets along the catkin, and (2) the anthers maturity leading to pollen dehiscence. The opening of florets along the catkin follows an acropetal pattern of development, where florets at the proximal end open first and

florets at the distal end open last. As each floret opens, filaments emerge at various times during anther's maturity.

Anthers are the male gametophytic structure and it is the location for the development of the pollen grains. As with many angiosperms, chestnut anthers are located at the apical tip of each filament, and are typically yellow in color (Dibuz, 1996). Mert and Soylu (2006) described fertile anthers as round, elliptical, and long, while sterile anthers were wide and lacked the normal layers of tissues that surround each anther; tapetum, endothecium, outer layer, and epidermal layer. Each anther consists of 2 lobes, where each lobe is subsequently divided in 2 pollen sacs.

The morphology of *Castanea* species pollen grains has been studied under light and electron microscopy. These studies revealed that chestnut pollen grains are prolate or sub-prolate in shape, with a polar axis (i.e. length) of 13-21 μm and an equatorial axis (i.e. width) of 8-14 μm (Beyhan and Serdar, 2008; Bounous et al, 1992b; Erdtman, 1966; Mert and Soylu, 2007). Each pollen grain has a reticulated surface with three germinal furrow regions (i.e. colpi) and one pore in the center of each colpus, resembling the colporate-type of pollen grains (Mert and Soylu, 2007). Each colpus region is long and narrow like channels, and it divides the surface of the pollen grain in three equal parts. Mert and Soylu (2007) compared pollen grains from male-sterile and male-fertile chestnut cultivars and found no significant difference among the cultivars studied.

Each chestnut pollen grain contains a pollen wall, two nuclei—one generative and one vegetative (Fernando et al, 2006), and the cytoplasm. Chestnut pollen grain walls consist of a thick intine layer and two thin exine layers; nexine and sexine. Fernando et al (2006) findings regarding the thickness of the intine layer was slightly different from that previously reported for

C. sativa species (Hyde and Adams, 1958). The exine layers are involved in anther maturity. The tapetum, the outer most layer of the exine, plays an important role in pollen development. Its major functions are to: (1) supply the nutrients necessary for the developing pollen grains, (2) release callase, an enzyme required for the breakdown of callose walls around the microspore, (3) secrete the precursor molecules of sporopollenin, a molecule necessary for the formation of pollen exine, and (4) supply pollenkit, a substance with a large number of proteins required for the formation of the pollen coat. Pollen grains develop from pollen mother cells (PMCs), also known as microspore mother cells, located in each lobe of the anthers. Each PMC originates after multiple mitotic events that occur within the cells of the sporogenous tissue, which is located underneath the tapetum (Shivanna, 2003). During microsporogenesis, PMCs undergo a series of synchronized meiotic divisions, which leads to the formation of four haploid microspores, and, ultimately, pollen grains differentiate from each microspore (Sauter, 1971). As the anthers' stomium split and the anthers dehydrate, mature pollen grains are released from the anthers (Shivanna, 2003).

In *Castanea* species, like in most angiosperms, each mature pollen grain is a haploid (n+n), two-celled structure (Fernando et al, 2006). One of the cells known as vegetative cell (n) leads the formation of the pollen tube, and the other cell known as generative cell (n) is essential for double fertilization (described in the Fertilization section below). The number of pollen grains released from a single chestnut anther varies among *Castanea* species and cultivars. Mert and Soylu (2006) observed major differences in pollen production among four *C. sativa* cultivars. They found that the number of pollen grains released from an anther per cultivar could vary from 120 to 5,200. Fernando et al. (2006) noted that chestnut pollen grains mature at different rates along the catkin, and that the proportion of mature pollen per catkin can vary from

one day to the next day. Once pollen grains dehisce from chestnut anthers, the typical yellow color of the anthers change from yellow to brown (*personal observation*, this dissertation). The browning of the anthers represents the end of male catkin anthesis or blooming (Fernando et al, 2006), and this color marks the end of the pollination period for a tree. This browning stage may serve as an indicator of the difference in phenology among chestnut species and cultivars.

Chestnut female inflorescences develop following a double cymose arrangement, in which each cyme gives rise to 2 or more symmetrically placed branches (i.e. dichasium) (Dibuz, 1996). As many anemophilus flowers of angiosperms, stamens remnants are present on chestnut female inflorescences (Percival, 1965). Descriptive reports regarding detailed morphology of chestnut female flowers are limited to representatives of *C. sativa* (Botta et al, 1995), *C. sativa* X *C. crenata* hybrids (Bounous et al, 1992a), and *C. mollissima* (Shi and Stosser, 2005). However, these reports provide a general view of the pistillate flowers of all *Castanea* species.

Pistillate flowers are borne at the base of each bisexual catkin and may appear singly or in clusters of 2 or more per catkin (Botta et al, 1995). The gynoecium, the structural unit of each female flower, consists of seven and occasionally eight carpels. Each carpel encloses the ovary, the placenta, and the ovules including the stigma and the styles; which collectively represent the pistil of a single flower. Pistillate flowers are part of a scaly involucre, where each involucre develops into a prickly bur. Each pistillate flower has three groups of styles, regardless if the involucre of the *Castanea* species develops into one, two or three nuts per bur. Each group of styles consists of 6 to 9 styles, where each style is a hollow, needle-shape, and cylindrical structure at the apex of each involucre. The color of the styles varies among cultivars from greenish white to light yellow (Bounous et al, 1992a). In 'Colossal', a European-Japanese chestnut hybrid, the central group of styles emerge early during female flower anthesis, while the

two lateral groups of styles develop about 5 to 7 days after the central group of styles have emerged (*personal observation*, this dissertation). At the tip of each style, an arrangement of cells collectively forms the stigma. Each stigma is needle shape with an aperture of approximately 50 micrometer in diameter, where long secreting cells release sticky fluids over its aperture (Shi and Xia, 2010). The period of stigma receptivity varies depending on environmental factors, the health of each tree, and the inflorescence traits of each species and cultivar. For temperate regions, like Michigan, the duration of chestnut stigma receptivity could last as long as 30 days (Soltesz et al, 1996).

Each female flower consists of one ovary that encloses the megasporangium, which will lead to the formation of ovules, and ultimately, the embryo sac. Each chestnut ovary contains 10-22 anatropus ovules that develop from the placental wall of the ovary (Botta et al, 1995; Nakamura 2001). Each ovule consists of two integuments and the nucellus. The two integument layers do not enclose the nucellus entirely, but there remains an opening resembling a channel known as the micropyle. The nucellus is the megasporangium, containing the megasporocyte or megaspore mother cell (MMC). During the process of megasporogenesis, the MMC undergoes meiosis resulting in 4 haploid cells, named megaspore and polar bodies. The megaspore becomes one mature ovule when the three polar bodies degrade prior to megagametogenesis. During megagametogenesis, the megaspore undergoes three mitotic divisions, and the embryo sac or megagametophyte is formed (Rieser and Fischer, 1993). The embryo sac of chestnuts consists of 7 cells with 8 nuclei (Botta et al, 1995). The development of these cells has been described to resemble the development of the Polygonum-type embryo sac, where the embyo sac consists of 3 antipodal nuclei, 2 synergid cells, 1 egg cell, and 1 central cell with 2 nuclei. These cells, and their polar orientation within the embryo sac are crucial components for each gynoecium to

engage in the process of double fertilization (described below). Botta et al (1995) observed that chestnut ovules in the ovary of the European cultivar 'Marron' were at various stages of megasporogenesis or megagametogenesis. Thus, although pollination maybe efficient, fertilization might not occur due to presence of immature ovules. Nakamura (1992) conducted observations in pistillate flowers of *C. crenata* to determine the optimal pollination time for Japanese chestnut. He noted that pistillate flowers undergo three consecutive developmental changes; (1) styles are fully extended, (2) styles stop to elongate and measure approximately 4 mm in length, and (3) scales of burs began to spread and change to spines. Earlier, Clapper (1954) noted that chestnut styles undergo at least two distinctive morphological changes that could serve as signs of stigma receptivity. He indicated that the color of the styles change from green to yellow and that the styles spread apart almost forming a right angle. Together, these changes are good indicators of the end of the pollination period. Furthermore, Shi and Xia (2010) described that the pollination period in *C. mollissima* lasts for at least 20 days, with an optimum of 11 to 15 days after the emergence of the stigmas.

Fertilization

Fertilization involves two broad processes, pollen-pistil interactions and double fertilization. In general, pollen-pistil interactions involve all the structural and biochemical changes that pollen and style orchestrate leading to double fertilization. The process of double fertilization signifies the reproduction of angiosperms. After the germination of a single pollen grain, the vegetative cell develops into the pollen tube leading the generative cell through the style and placing the generative cell in proximity to the micropyle end of the embryo sac. Before the generative cell reaches the embryo sac, the haploid generative cell undergoes one cycle of mitosis creating two haploid cells known as sperm cells. Upon entrance of the vegetative cell and

the sperm cells through the micropyle, the vegetative cell nucleus and one of the synergid cells in the embryo sac degrades. The degradations of these cells, allows the first fertilization event, where one sperm cell unites with the egg cell resulting in the zygote (2N). The second fertilization event occurs when the remaining sperm cell comes together with the diploid central cell resulting in the endosperm (3N).

There are only a limited number of reports regarding the process of fertilization in chestnuts. Nakamura (2001) studied pollen tube growth in relationship to fertilization to understand ovule growth and degradation. He noticed that in *C. crenata*, only one ovule enlarges while the other 17-22 ovules degenerate. On average, the number of pollen tubes reaching the nucelli of the ovules was 6 out of 11 tubes seen at any given time within the style. He determined that under optimal conditions, the trajectory of a single chestnut pollen tube to the nucellus of an ovule takes 14 days. In European chestnut cultivars, fertilization occurs between 12 to 15 days after blooming. At this time, the nucleus of the endosperm should be visible under light microscopy (Botta et al, 1995).

Upon fertilization, pollinated flowers transform into burs enclosing 1 to 3 or more nuts. Burs are green prickly structures characteristic of all *Castanea* species. Generally, burs will split open after seed maturity, which allows the release of the seeds or nuts. On some trees, mature closed burs may fall from trees without the release of the nuts. The fracture of the burs could either be a single crack resembling a clam-shape or multiple cracks resembling a star-shape. The shape of the facture of burs has been used as a taxonomic to distinguish between chinquapin and true chestnut species. All chinquapin species consist of clam-shape mature burs, while true chestnut species consist of star-shape mature burs.

Few studies have been performed to correlate successful fertilization and bur development. Nakamura (2001) described three visible morphological changes between fertilized and non-fertilized ovules. These visible changes included the transformation of flower scales into prickly spikes, the casing of the pistil by the enlargement of the bur, and the variation in weight of the bur. He indicated that the external appearance of all flowers will change from scales to soft spines, and from soft spines to prickly spikes (i.e. bur), regardless if ovules within each flower were fertilized or not fertilized. However, the styles of non-fertilized flowers remained exposed to the atmosphere, and the weight of the bur from non-fertilized flower will cease to increase as soon as 10 days after pollination. Nakamura (2001) noticed that burs developing from these non-fertilized flowers might change from yellow to brown and abscise naturally. As the weight of the burs resulting from flowers with fertilized ovules increases, a cascade of synchronized events occurring inside fertilized ovules leads to the formation and development of the embryo and the endosperm (see below). Together, the embryo and the cotyledons are considered as the fruit or nut.

Embryo Formation and Development

The formation of the embryo is the culmination of the processes of pollination and fertilization. Seed formation and development is the beginning of a new individual, and the perpetuation of a species. Embryos of chestnuts are globular, heart-shaped, and then torpedo-shape. Endosperm becomes cellular at the globular stage of the embryo. Two cotyledons replace the endosperm about 20 to 35 days after fertilization (Botta et al, 1995; Nakamura, 2001). During seed development, the integuments within the ovary become the seed coat, or the pellicle of the chestnut fruit. None of these morphological changes could be seen in non-fertilized ovules, where the resulting empty burs will consist of flat shells filled with fiber and aborted ovules.

Shi and Xia (2010) clearly summarized the main causes for low yields due to a high number of empty cupules or burs. In their summary, they included at least one reason due to each of these processes; pollination, fertilization, and embryo development. A few of the important topics be considered during the establishment and maintenance of chestnut orchards are (1) asynchrony between pollen dehiscence and stigma receptivity (pollination), abnormal formation of male or female gametophyte (pollination), (2) incompatible pollen-pistil interaction during fertilization, and (3) embryo abortion.

CURRENT ASPECTS OF CHESTNUT POLLINATION IN MICHIGAN

Poor pollination in many Michigan chestnut orchards throughout the state appears responsible for serious yield losses and delays in producing substantial crops (Fulbright and Mandujano, 2002). On-farm yields of the commercial cultivar 'Colossal' can be severely limited and disappointing when compared with the outstanding yields obtained in cultivar tests performed at research stations across the state. In most cases, 'Colossal' still produces more chestnuts than other cultivars or seedlings on the same farm, but only about 10-50 percent of the amount expected based on yield trials. Cultivar tests at two research stations indicated that a ‘Colossal’ tree should be producing between 5 – 10 kilograms (kg) per tree by the sixth growing season and could be producing as much as 40 kg per tree by the tenth season (Fulbright and Mandujano, 2002). At both research stations the number of pollinizer trees ranged from 20 to 30 trees per every 100 ‘Colossal’ trees, and these pollinizer trees represented various chestnuts cultivars where its pollen deshiced from early June until late August (*personal communication*, Mario Mandujano). Observations have lead researchers to hypothesize that a lack of pollination is the second most important reason for the observed yield limitation (the first being damaging spring

frosts). Flower production is not the problem as by the third growing season ‘Colossal’ is typically producing dozens of female flowers and by the sixth growing season, well over a hundred flowers are available for pollination and nut set. However, instead of nuts, growers find 90 percent of empty burs or non-pollinated nuts (i.e. flat nuts) at harvest. Two possible explanations as to why poor pollination is limiting yields on-farms are that (1) the pollinizer trees could not be producing pollen in synchrony with ‘Colossal’ trees and its flowers, or (2) the pollinizer trees could be too young to produce pollen or could not be producing enough amount of pollen to reach the large amount of ‘Colossal’ trees and its flowers.

Like most nut trees, all species of chestnuts are monoecious and self-incompatible (Clapper, 1954; Solignat, 1958), thus, requiring out-crossing for nut production (Miller 2003). The cultivar ‘Colossal’ is pollen sterile and cannot pollinize other chestnut cultivars, but other cultivars should be able to pollinize ‘Colossal’. The cultivars ‘Nevada’, ‘Silverleaf’, ‘Okei’ and ‘Fowler’ are known to successfully pollinize ‘Colossal’ in the Central Valley of California and in southeastern Washington State. In Michigan, several orchards established since 1997, where ‘Colossal’ was planted only with ‘Nevada’, have produced chestnuts, but at lower yields than expected (Fulbright and Mandujano, 2001; Fulbright and Mandujano, 2002). It is clear that other pollinizers must be found and placed in the orchards.

At SWMREC in Benton Harbor, the requirements for successful pollination of 'Colossal' are being met by one or more of the 20 cultivars planted in the plot, but it is not known which trees are contributing the pollen. This knowledge would help the chestnut industry find pollinizers for ‘Colossal’. Therefore, genetic marker technology, the ability to separate parents in a cross and define progeny, can play a pivotal role in our understanding of the process of pollination.

THESIS GOALS

Because of the mixture of cultivars and hybrids in Michigan orchards and the variability in nut production among orchards with European-Japanese and Chinese cultivars, cultivar identification and pollination efficiency are of main focus of my dissertation.

My goals for this dissertation are to 1) identify chestnut cultivars currently growing in Michigan using Simple Sequence Repeats (SSRs) as genetic markers, and 2) improve our understanding of chestnut reproduction including reproductive phenology, pollination, and fruit-set using genetic analysis of parents and progeny.

LITERATURE CITED

LITERATURE CITED

Ahmad, R., Ferguson, L., and S M. Southwick. 2003. Identification of pistachio (*Pistacia vera* L.) nuts with microsatellite markers J. Am. Soc. Hort. Sci. 128:898–903.

Akerman, J D. 2000. Abiotic pollen and pollination: ecological, functional, and evolutionary perspectives In: Pollen and Pollination, Dafni, A, House, M, and E, Pacini (eds.) Springer Wien, New York.

Akkak, A., Boccacci, P., and D T. Marinoni. 2010. Cross-species amplification of microsatellite markers in *Castanea* spp. and other related species. Acta Hort. 866:195–201.

Aldrich, P R., Jagtap, M., Michler, C H., and J. Romero-Severson. 2003. Amplification of North American red oak microsatellite markers in European white oaks and Chinese chestnut. Silvae Genet. 52:176–179.

Anagnostakis, S L. 1999. Cultivars of Chestnuts, in: 90th Annual Report of the Northern Nut Growers Association, Northern Nut Growers Assoc., Inc. Cornell, New York, USA.

Anagnostakis, S L. 2010. Identification of Chestnut Trees. URL www.ct.gov/caes

Aranzana, M J., Abbassi, E K., Howad, W., and P. Arús. 2010. Genetic variation, population structure and linkage disequilibrium in peach commercial varieties. BMC Genetics 11:69.

Barreneche, T., Casasoli, M., Russell, K., Akkak, A., Meddour, H., Plomion, C., Villani, F., and A. Kremer. 2004. Comparative mapping between *Quercus* and *Castanea* using simple sequence repeats (SSRs). Theor. Appl. Genet. 108:558–566.

Bassi, G., and H. Craddock. 1998. Poster number 35, Performance and description of the introduced chestnut cultivar 'Colossal' in Cuneo province, Northwest Italy in: 2nd International Chestnut Symposium (Abstract and List of Participants), Salesses, G (ed.) ISHS, INRA.

Benedek, P. 1996. Insect pollination of fruit crops, in: Floral biology of temperate zone fruit trees and small fruits. Nyeki, J and M, Soltesz (eds.) Akademiai Kiado, Budapest.

Beyhan, N., and U. Serdar. 2008. *In vitro* pollen germination and tube growth of some European chestnut genotypes (*Castanea sativa* Mill.). Fruits 64:157–165.

Boccacci, P., Akkak, A., and R. Botta. 2006. DNA typing and genetic relations among European hazelnut (*Coryllus avellana* L.) cultivars using microsatellite markers. Genome 49:598–611.

Boccacci, P., Akkak, D T., Marinoni, D., Bounous, G., and R. Botta. 2004. Typing European chestnut (*Castanea sativa* Mill.) cultivars using oak Simple Sequence Repeat Markers. HortSci. 39:1212–1216.

Botta, R., Akkak, A., Guaraldo, P., and G. Bounous. 2005. Genetic characterization and nut quality of chestnut cultivars from Piemonte (Italy). Acta Hort. 693:395–401.

Botta, R., Vergano, G., Me, G., and R. Vallania. 1995. Floral biology and embryo development in chestnut (*Castanea sativa* Mill.). HortScience 30:1283–1286.

Bounous, G., Craddock, J H., Peano, C., and P. Salarin. 1992a. Phenology of blooming and fruiting habits of Euro-Japanese hybrid chestnut. Proceedings of the International Chestnut Conference. West Virginia University Press, Morgantown, WV, USA.

Bounous, G., Paglietta, R., and C. Peano. 1992b. Methods for observing chestnut pollen viability, germinability, and pollen tube growth. Proceedings of the International Chestnut Conference. West Virginia University Press, Morgantown, WV, USA.

Bricher, A G., and W H. Bricher. 2000. Encyclopedia of fruit trees and edible flowering plants in Egypt and the Subtropics. The American University in Cairo Press, Cairo, New York.

Brickell, C D., Alexander, C., David, J C., Hetterscheid, W L A., Leslie, A C., Malecot, V., and X. Jin (eds.). 2009. International Code of Nomenclature for Cultivated Plants—Eight edition. Scripta Horticulturae 10, ISHS, Drukkerij Geers, Gent-Oostakker, Belgium.

Buck, E J., Hadonou, M., James, J., Blakesley, D., and K. Russel. 2003. Isolation and characterization of polymorphic microsatellites in European chestnut (*Castanea sativa* Mill.) Mol. Ecol. Notes 3:239–241.

Buteler, M I., LaBonte, D R., Jarret, R L., and R E. Macchiavelli. 2002. Microsatellite-based paternity analysis in polyploid sweetpotato. J. Amer. Soc. Hort. Sci. 127:392–396.

Clapper, R B. 1954. Chestnut breeding, techniques and results. J. Hered. 45:107–114, 201–208.

Cipriani, G., Spadotto, A., Jurman, I., Di Gaspero, G., Crespan, M., Meneghetti, S., Frare, E., Vignani, R., Cresti, M., Morgante, M., Pezzotti, M., Pe, E., Policriti, M., and R. Testolin. 2010. The SSR-based molecular profile of 1005 grapevine (*Vitis vinifera* L.) accessions uncovers new synonymy and parentages, and reveals a large admixture amongst varieties of different geographic origin. Theor. Appl. Genet. 121:1569–1585.

Costa, R., Valdivieso, T., Marum, L., Borges, O., Soeiro, J., Assuncao, A., Fonseca, L., Matos Soares, F., Sequeira, J., and P. Correia. 2005. Characterisation of traditional Portuguese chestnut cultivars by nuclear SSRs. Acta Hort. 693:437–440.

Conedera, M., Krebs, P., Tinner, W., Pradella, M., and D. Torriani. 2004. The cultivation of *Castanea sativa* (Mill.) in Europe, from its origin to its diffusion on a continental scale.

Veget. Hist. Archaeobot. 13:161–179.

De Oliveira, D., Gomes, A., Ilharco, F A., Manteigas, A M., and J. Ramalho. 2001. Importance of Insect Pollinators for the Production in the Chestnut, *Castanea sativa*. Acta Hort. 561:269–273.

Dibuz, E. 1996. Morphological characteristics of flowers and fruits, in: Floral biology of temperate zone and fruit trees and small fruits. Nyeki, J. and M, Soltesz (eds.) Akademiai Kiado, Budapest.

Dinis, L T., Ramos, S., Gomes-Laranjo, J., Peixoto, F., Costa, R., Vallania, R., and R. Botta. 2009. Phenology and reproductive biology of cultivar 'Judia' (*Castanea sativa* Mill). Acta Hort. 866:169–174.

Donis-Gonzales, I. 2008. Management of microbial decay in fresh and peeled chestnuts in Michigan. (Thesis) Department of Plant Pathology, Michigan State University, East Lansing, Michigan.

Ertman, G. 1966. Pollen morphology and plant taxonomy, Angiosperms, An Introduction to Palynology I. Hafner Publishing Company, New York and London.

Fernando, D.D., Richards, J L., and J R. Kikkert. 2006. In vitro germination and transient GFP expression of American chestnut (*Castanea dentata*) pollen. Plant Cell Rep. 25:450–456.

Frankel, R., and E. Galun. 1977. Biotic vectors In: Pollination mechanisms, reproduction, and plant breeding, Frankel, R, and B, Dagan (eds.) Springer-Verlag, New York.

Foroni, I., Woeste, K., Monti, L M., and R. Rao. 2007. Identification of 'Sorrento' walnut using simple sequence repeats (SSRs). Genet. Resour. Crop Evol. 54:1081–1094.

Fulbright, D W. 2011. Common Chestnut Cultivars Grown in Michigan in: Michigan State University's Rogers Reserve. Fulbright, D W (ed.) URL http://www.rogersreserve.org

Fulbight, D W., and M. Mandujano. 1999. Evaluation of the chestnut cultivar 'Colossal' in Michigan. Annual Report Northern Nut Growers Assoc. 90:48–55.

Fulbright, D W., and M. Mandujano. 2001. Pollination of new Michigan chestnut orchards in 2000 and 2001, in: 92nd Annual Report of the Northern Nut Growers Association, Annual Report Northern Nut Growers Assoc.,Inc. Cornell, New York, USA.

Fulbright, D W., and M. Mandujano. 2002. Establishing chestnut orchards in Michigan. Annual Report of the Northern Nut Growers Association, NNGA,Inc. Cornell, New York, USA.

Gaoping, W., Qing, Y., Kail Z., and W M. Ciesla. 2001. Factors affecting production of Chinese Chestnut in Xinxian County, Henan Province, China. The Forest Chronicle 77: 839–845.

Gouta, H., Ksia, E., Buhner, T., Moreno, M A., Zarrouk, M., Mliki, A., and Y. Gorgocena. 2010. Assessment of genetic diversity and relatedness among Tunisian almond germplasm using SSR markers. Hereditas 147: 283–292.

Gerber, S., Mariette, S., Streiff, R., Bodenes, C., and A. Kremer. 1999. Chapter 4 in: Which DNA Marker for Which Purpose? Final Compendium of the Research Project Development, optimisation and validation of molecular tools for assessment of biodiversity in forest trees in the European Union DGXII Biotechnology FW IV Research Programme Molecular Tools for Biodiversity. Gillet, E M. (ed.). URL http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm

Gillet, E M. 1999. Chapter 2 in: Which DNA Marker for Which Purpose? Final Compendium of the Research Project Development, optimisation and validation of molecular tools for assessment of biodiversity in forest trees in the European Union DGXII Biotechnology FW IV Research Programme Molecular Tools for Biodiversity. Gillet, E M. (ed.). URL http://webdoc.sub.gwdg.de/ebook/y/1999/whichmarker/index.htm

Gobbin, D., Leandro, H., Conza, L., Jermini, M., Gessler, C., and M. Conedera. 2007. Microsatellite-based characterization of the *Castanea sativa* cultivar heritage of southern Switzerland. Genome 50: 1089–1103.

Gold, M., Cernusca, M M., and L D. Godsey. 2006. Competitive market analysis: chestnut producers. HortTech 16:360–369

Grauke, L J., Iqbal, M J., Reddy, A S., and T E. Thompson. 2003. Developing microsatellite DNA markers in pecan. J. Amer. Soc. Hort. Sci. 128:374–380.

Graves, A H. 1992. Illustrated guide of trees and shrubs: A handbook of the woody plants of the Northern United States and adjacent Canada. Dover Publications Inc. Mineola, New York.

Han, J C., Wang, DJ., Liu, Q.X., and X Y. Zhang. 2007. Genetic diversity of Chinese chestnut (*Castanea mollissima*) in Hebei. Acta Hort. 760:575–577.

Hasegawa, Y., Suyama, Y., and K. Seiwa. 2009. Pollen donor composition during the early phases of reproduction revealed by DNA genotyping of pollen grains and seeds of *Castanea crenata*. New Phytologist 182: 994–1002.

Hayden, M J., Nguyen, T M., Waterman, A., McMichael, G L., and K J. Chalmers. 2008. Application of multiplex-ready PCR for fluorescence-based SSR genotyping in barley and wheat. Mol Breed. 21:271–281.

Hayden, M J., Tabone, E T., and E D. Mather. 2009. Development and assessment of simple PCR markers for SNP genotyping in barley. Theor. Appl. Genet. 119:939–951.

Hormaza, JI., and A. Wunsch. 2007. Pistachio in: Fruits and Nuts—Genome mapping and molecular breeding in plants. Kole, C (ed.) Spring-Verlag, Berlin.

Hozova, L., Jankovsky, L., Akkak, A., Marioni, D T., Botta, R., and J. Smerda. 2009. Preliminary study of the genetic structure of a chestnut population in the Czech Republic based on SSR Analysis. Acta Hort. 815:43-49.

Huang, H., Fenny, D., and T. Kubisiak. 1998. Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut (*Fagaceae*). Am. J. Bot. 85:1013–1021.

Hyde, H A., and K F. Adams. 1958. An atlas of airborne pollen grains. MacMillan and Co LTD, St. Martin's Press, New York.

Inoue, E., Ning, L., Hara, H., Ruan, S.A., and H. Anzai. 2009. Development of Simple Sequence Repeat markers in Chinese chestnut and their characterization in diverse chestnut cultivars. J. Am. Soc. Hort. Sci. 134:610–617.

Isagi, Y., Kanazashi, T., Suzuki, W., Tanaka, H., and T. Abe. 2004. Highly variable pollination patterns in *Magnolia obovata* revealed by microsatellite paternity analysis. Int. J. Plant Sci. 165:1047–1053.

Karimi, R., Ershadi, A., Vahdati, K., and K. Woeste. 2010. Molecular characterization of Persian walnut populations in Iran with microsatellite markers. HortSci. 45:1403–1406.

Kelley, K M., and B K. Behe. 2002. Chef's perceptions and uses of 'Colossal' chestnuts. HortTechnology 12:172–175.

King, R A., Harris, S L., Karp, A., and J H A. Barker. 2010. Characterisation and inheritance of nuclear microsatellite loci for use in population studies of allotetraploid *Salix alba—Salix fragilis* complex. Tree Genet.Genomes 6:247–258.

Lewers, K S., Styan, S M N., Hokanson, S C., and N V. Bassil. 2005. Strawberry GenBank-derived genomic simple sequence repeat (SSR) markers and their utility with strawberry, blackberry, and red and black raspberry. J. Amer. Soc. Hort. Sci. 130:102–115.

Li, X Q., Zhou, X Y., Zhou, J., Dodson, J., Zhang H B., and X. Shang. 2007. The earliest archaeobiological evidence of the broadening agriculture in China recorded at Xishanping site in Gansu Province. Science in China Series D-Earth Sciences 50:1707-1714.

Liu, W., Kang, M., Tian, H., and H. Huang. 2013. A range wide geographic pattern of genetic diversity and population structure of *Castanea mollissima* populations inferred from nuclear and chloroplast microsatellites. Tree Genet. Gen. 9:975–987.

Lusini, I., Velichkov, I., Pollegioni, P., Chiocchini, F., Hinkov, G., Zlatanov, T. Cherubini, M.,

and C. Mattioni. 2014. Estimating the genetic diversity and spatial structure of Bulgarian *Castanea sativa* populations by SSRs: implications for conservation. Conserv.Genet. 15:283–293.

Marinoni, D., Akkak, A., Bounous, G., Edwards, K.J., and R. Botta. 2003. Development and characterization of microsatellite markers in *Castanea sativa* (Mill.) Mol. Breed. 11:127–136.

Marsico, T.D., Hellmann, J.J., and J. Romero-Severson. 2009. Patterns of seed dispersal and pollen flow in *Quercus garryana* (*Fagaceae*) following post-glacial climatic changes. J. Biogeography 36:929–941.

Martin, M.A., Alvarez, J.B., Mattioni, C., Cherubini, M., Villani, F., and L M. Martin. 2009a. Identification and characterization of traditional chestnut varieties of southern Spain using morphological and simple sequence repeat (SSRs) markers. Ann. Appl. Biol. 154:389–398.

Martin, M.A., Alvarez, J.B., Mattioni, C., Cherubini, M., Villani, F., and L M. Martin. 2009b. Microsatellite-based characterization of traditional chestnut cultivars of Italy. Acta Hort. 866:157–162.

Martin, M.A., Mattioni, C., Cherubini, M., Taurchini, D., and F. Villani. 2010a. Genetic characterisation of traditional chestnut varieties in Italy using microsatellites (simple sequence repeats) markers. Ann. Appl. Biol. 157:37–44.

Martin, M.A., Mattioni, C., Cherubini, M., Taurchini, D., and F. Villani. 2010b. Genetic diversity in European chestnut populations by means of genomic and genic microsatellite markers. Tree Genet. Gen. 6:735–744.

Martin, M A., Mattioni, C., Molina J R., Alvarez J B., Cherubini, M., Herrera M A., Villani, F., and L M. Martin. 2012. Landscape genetic structure of chestnut (*Castanea sativa* Mill.) in Spain. Tree Genet. Gen. 8: 127–136.

Mattioni, C., Martin, M A., Pollegioni, P., Cherubini, M., and F, Villani. 2013. Microsattelite markers reveal a strong geographical structure in European populations of *Castanea sativa* (*Fagaceae*): evidence for multiple glacial refugia. Am. J. of Botany 100: 951–961.

Medina-Mora, C M. 1999. Genetic variation in strains of *Clavibacter michiganensis* subsp. *michiganensis* and the development of bird's eye fruit lesions on tomatoes. Thesis (M.S.)**,** Michigan State University. Dept. of Botany and Plant Pathology, East Lansing, Michigan.

Mert, C., and A. Soylu. 2006. Flower and stamen structures of male-fertile and male-sterile chesntut (*Castanea sativa* Mill.) cultivars. J. Amer. Soc. Hort. Sci. 131:752–759.

Mert, C., and A. Soylu. 2007. Morphology and anatomy of pollen grains from male-fertile and male-sterile cultivars of chestnut (*Castanea sativa* Mill.) J. Hort. Sci. Biotech. 82:474–480.

Meehan, T. 1879. On sex in *Castanea americana*. Proceedings of the Academy of Natural Sciences of Philadelphia. 31:165–167.

McCleary, T., McAllister, M., Coggeshall, M., and J. Romero-Severson. 2013. EST-SSR markers reveal synonymies, homonymies and relationships inconsistent with putative pedigrees in chestnut cultivars. Genet. Resour. Crop Evol. 60:1209–1222.

McKay, J W. 1942. Self-sterility in the Chinese chestnut. Proc. Am. Hort. Sci. 41:156–160.

McKay, J W. 1972. Pollination of chestnut by honey-bees. North. Nut Growers Asson. Ann. Rpt. 63:83–86.

Miller, G., Miller, D.D., and, R A. Jaynes. 1996. Chestnuts, in: Fruit Breeding. Janick, J and J N, Moore (eds). Volume III. Nuts. John Wiley and Sons, Inc. New York.

Miller, G. 2003. Chestnuts, in: A guide to nut tree culture in North America. Fulbright, D (ed.). Volume 1. Northern Nut Growers Assn., Inc. Cornell, New York.

Milgroom, M G. 1995. Population biology of the chestnut blight fungus, *Cryphonectria parasitica*. Can. J. Botany. 73:S311-S319.

Nakamura, M. 1992. Elongation of styles and optimum pollination tin in Japanese chestnut (*Crenata sativa* Sieb. & Zucc.). (Summary in English) J. Japan. Soc. Hort. Sci. 61:265–271.

Nakamura, M. 2001. Pollen tube growth and fertilization in Japanese chestnut (*Castanea crenata* Sieb. & Zucc.) J. Japan. Soc. Hort. Sci. 70:561–566.

Nishida, M. 1983. The emergence of food production in Neolithic Japan. J. Antro. Archae. 2:305–322.

Nishio, S., Iketani, H., Fujii, H., Yamamoto, T., Terakami, S., Takada, N., and T. Saito. 2014. Use of population structure and parentage analyses to elucidate the spread of native cultivars of Japanese chestnut. Tree Gen. Genom. 10:1171–1180.

Nishio, S., Yamamoto, T., Terakami, S., Sawamura, Y., Takada, N., and T. Saito. 2011. Genetic diversity of Japanese chestnut cultivars assessed by SSR markers. Breed. Sci. 61:109–120.

Ohata, T., and T. Sato. 1961. Studies on the pollination of chestnut trees. Bulletin Nat. Inst. Agri. Sci. Ser E 9:185–193.

Paillet, F L. 2002. Chestnut: history and ecology of a transformed species. J. Biogeography 29: 1517–1530.

Pereira-Lorenzo, S., Diaz-Hernandez, M B., and A M. Ramos-Cabrer. 2006. Use of highly discriminating morphological characters and isozymes in the study of Spanish chestnut cultivars. J. Amer. Soc. Hort. Sci. 131:770–779.

Pereira-Lorenzo, S., Fernandez-Lopez, J., and J. Moreno-Gonzalez. 1996. Variability and grouping of northwestern Spanish chestnut cultivars.II. Isozymes traits. J. Amer. Soc. Hort. Sci. 121:190–197.

Pereira-Lorenzo, S., Ramos-Cabrer, A M., Gonzalez-Diaz, A J., and M B. Diaz-Hernandez. 2008. Genetic assessment of local apple cultivars from La Palma, Spain, using simple sequence repeats (SSRs). Sci. Hort. 117:160–166.

Pereira-Lorenzo, S., Lourenço Costa, RM., Ramos-Cabrer, A M., Ciordia-Ara, M., Marques Ribeiro, C M., Borges, O., and T. Barreneche. 2011. Chestnut cultivar diversification process in the Iberian Peninsula, Canary Islands, and Azores. Genome 54:301–310.

Percival, M. 1965. Floral Biology. Pergamon Press, New York, USA.

Pijut, P M., Woeste, K E., Vengadesan, G., and C H. Michler. 2007. Technological advances in temperate hardwood tree improvement including breeding and molecular marker applications. In Vitro Cell. Dev. Biol. Plant 43:283–303.

Pollegioni, P., Woeste, K., Scarascia Mugnozza, G., and M E. Malvolti. 2009. Retrospective identification of hybridogenic walnut plants by SSR fingerprinting and parentage analysis. Mol Breed. 24:321–335.

Powell, W., Machray, G C., and J. Povan. 1996a. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. 1:215–222.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., and A. Rafalski. 1996b. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breed. 2:225–238.

Proctor, M., and P. Yeo. 1973. The pollination of flowers. Collins, St. James's Place, London.

Queijeiro, J.M., De la Montana, J., and M. Miguez. 2006. Identification and morphological description of cultivars of chestnut (*Castanea sativa* Mill) of the region of Verin-Monterrei (Ourense, Spain). J. Am. Pom. Soc. 60:37–45.

Rafalski, J A., and S V. Tingey. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends Genet. 9:275–280.

Reiser, L., and L. Fischer. 1993. The Ovule and the Embryo Sac. The Plant Cell 5:1291–1301.

Rupp, J V., Mangolin, C A., Scapim, C A., and M F. Pires da Silva Machado. 2009. Genetic structure and diversity among sweet corn (su1-germplasm) progenies using SSR markers. Maydica 54:125–132.

Rutter, P A. 1996. Chestnut pollinator's guide. Bulletin 1, Badgersett Research Corporation, Pensylvania, USA.

Sauter, J J. 1971. Nucleus and cytoplasm in microsporogenesis, in: Pollen: development and physiology. Heslop-Harrison, J (ed.) Butterworths, London.

Selvaraj, C I., Nagarajan, P., Thiyagarajan, K., Bharathi, M., and R. Rabindran. 2011. Identification of Microsatellite (SSR) and RAPD Markers Linked to Rice Blast Disease Resistance gene in Rice (*Oryza sativa* L.). African J. Biotech. 10:3301–3321.

Shi, Z., and L. Xia. 2010. Stigmatic morphology of Chinese chestnut (*Castanea mollissima* Blume). HortSci. 45:981–983.

Shivanna, K R. 2003. Pollen biology and biotechnology. Science Publishers, Inc. Enfield, New Hampshire, USA.

Sisco, P. 2011. Chestnut Identification, in: The American Chestnut Foundation URL http:// www.mindspring.com/~psisco/www/overview.html

Solar, A., Podjavorsek, A., and F. Stampar. 2005. Phenotypic and genotypic diversity of European chestnut (*Castanea sativa* Mill.) in Slovenia —opportunity for genetic improvement. Gen. Res. Crop Evol. 52:381–394.

Soltesz, M. 1996. Requirements for successful fruit set in orchards, in: Floral biology of temperate zone fruit trees and small fruits. Nyeki, J, and M, Soltesz (eds.) Akademiai Kiado, Budapest.

Soltesz, M., Nyeki, J., and Z. Szabo. 1996. Receptivity of sexual organs, in: Floral biology of temperate zone fruit trees and small fruits. Nyeki, J, and M, Soltesz (eds.) Akademiai Kiado, Budapest.

Solignat, G. 1958. Observation on the biology of the chestnut. Ann. Inst. Nat. Rech. Agron. 8:31–58.

Stafne, E T., Clark, J R., Weber, C A., Graham, J., and K S. Lewers. 2005. Simple sequence repeat (SSR) markers for genetic mapping of raspberry and blackberry. J. Amer. Soc. Hort. Sci. 130:722–728.

Stanley, R G., and H F. Linskens. 1974. Pollen: biology, biochemistry, management. Springer-Verlag, New York, Heidelberg, Berlin.

Tabbener, H E., and J E. Cottrell. 2003. The use of PCR based DNA markers to study paternity of poplar seedlings. Forest Eco. Manag. 179:363–376.

Tanaka, K., Tsumura, V., and T. Nakamura. 1999. Development and polymorphism of microsatellite markers for *Fagus crenata* and the closely related species, *F. japonica*. Theor. Appl. Genet. 99:11–15.

Tanaka, T., Yamamoto, T., and M. Suzuki. 2005. Genetic diversity of *Castanea crenata* in Northern Japan assessed by SSR markers. Breed. Sci. 55:271–277.

Tantasawat, P., Trongchuen, J., Prajongjai, T., Jenweerawat S., and W. Chaowiset. 2011. SSR analysis of soybean (*Glycine max* (L.) Merr.) genetic relationship and variety identification in Thailand. Australian J. Crop Sci. 5:283–290.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucl. Acids Res. 17:6463–6471.

Thomas, M R., and N S. Scott. 1993. Microsatellite repeats in grapevine reveal DNA polymoprhisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. 86:985–990.

UPOV. 1988. Guidelines for the conduct of tests for distinctness, homogeneity and stability (Chestnuts). International Union for the Protection of New Varieties of Plants. www.upov.int/en/publications/tg_rom/tg_index.html

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

Villani, F., Sansotta, A., Cherubini, M., Cesaroni, D., and V. Sbordoni. 1999. Genetic structure of natural populations of *Castanea sativa* in Turkey: evidence of a hybrid zone. J. Evol. Biol. 12:233–244.

Wang, Y., Kang, M., and H W. Haung. 2008. Microsatellite loci transferability in chestnut. J. Am. Soc. Hort. Sci. 133:692–700.

Warmund, M R., Elmore, J R., Adhikari, K., and S. McGraw. 2011. Descriptive sensory analysis and free sugar contents of chestnut cultivars grown in North America. J. Sci. Food Agric. 91:1940–1945.

Yamamoto, T., Kimura, T., Sawamura, Y., Manabe, Kotobuki, T., Hayashi, T., Ban, Y., and N. Matsuta. 2002. Simple sequence repeats for genetic analysis of pear. Euphytica 124:129–137.

Yamamoto, T., Kotobuki, K., Matsuta, N., Suzuki, M., and T. Hayashi. 2003. Characterization of simple sequence repeats in Japanese chestnut. J. Hort. Sci. Biotech. 78:197–203.

Zander, R H. 2000. Identification of Chestnuts (*Castanea*) species. URL http://www.mobot.org/plantscience/resbot/ches/chkey.htm

Zhang, D., Mischke, S., Johnson, E S., Phillips-Mora, W., and L. Meinhardt. 2009. Molecular characterization of an international cacao collection using microsatellite markers. Tree Genet. Genomes 5:1–10.

IDENTIFICATION OF CHESTNUT CULTIVARS AND PROGENY USING SSRs

ABSTRACT

Chestnut germplasm in Michigan consists of a mixture of introduced cultivars and hybrids from *Castanea* species originating from Asia (*C. crenata*), Europe (*C. sativa*), and Japan (*C. mollissima*), the interspecific crosses among trees of *Castanea* species and the naturalized population of trees from the American chestnut species (*C. dentata*), and plantings of seedlings, cultivars and hybrids in orchards. Besides remnants of the naturalized population of American chestnut in the state, historically, the chestnut population in Michigan increased in number due to the introduction of trees with unknown or putative pedigrees. The trees introduced to orchards were primarily selected by chestnut growers and amateurs, and were mainly planted for its favorable characteristics in the commercialization chestnuts. Two of the main characteristics why which these trees were introduced were resistance to chestnut blight caused by the fungal pathogen, *Cryphonectria parasitica*, and high nut quality and productivity. Presently, the identification and denomination of cultivars growing in Michigan has not been monitored, and the genetic diversity of the chestnut population has not been assessed due to the complexity of Michigan's germplasm. Leaf samples (n=109) representing 11 chestnut cultivars were selected in this study to explore the use of 5 simple sequence repeats (SSR) markers as a method of cultivar identification. All 5 SSR loci (CsCAT1, CsCAT2, and CsCAT16, EMCs15, and QrZAG75) selected for the identification of the 11 chestnut cultivars and hybrids were polymophic, where ten or more alleles were observed per loci (range: 10-19 alleles). The SSR profiles arising from the combination of alleles across all loci resulted in 55 genotypes; where all the samples from the chestnut cultivars 'Colossal' (34 out of 109 samples) and 'Everfresh' (5 out of 109 samples) resulted in 1 genotype per cultivar. High levels of allelic diversity were observed within samples

of each cultivar with the exception of samples of ‘Colossal’ and ‘Everfresh’, which seem to be truly clonally propagated. Unique SSR alleles were observed for 5 of the 11 cultivars (‘Colossal’, ‘Benton Harbor’, ‘Nevada’, and ‘Okei’) included in the study, where CsCAT2 locus resulted to identify the majority of unique alleles. A total of 9 unique alleles were identified for the cultivars included in this study, and the range of unique alleles observed was from 88 to 210 basepairs in length. The presence of unique SSR alleles and the high level of genetic diversity accomplished by the combination of SSR profiles across all loci per cultivar enable the use of these 5 SSR markers (CsCAT1, CsCAT2, and CsCAT16, EMCs15, and QrZAG75) as a resourceful methodology for chestnut identification.

In the summer of 2010, a cross-pollination study was performed, where a single ‘Okei’ (*C. crenata* × *C. pumila* var. *pumila*) tree, and a single ‘Benton Harbor’ (*C. mollissima*) tree, were pollen sources to four ‘Colossal’ (*C. sativa* × *C. crenata*) trees. The European × Japanese cultivar was ‘Colossal’ selected as the materal tree for the nuts (*i.e*. F1 progeny) of this cross because of its relative abundance in Michigan orchards. In this study, a total of 70 nuts were harvested from the 180 monitored female ‘Colossal’ flowers. Paternity was tested by inserting a bone marrow sampling tool through the shell of each nut and removing a small amount of kernel tissue. The DNA of the kernel tissue sampled via bone marrow was isolated and genotyped with the EMCs15 locus. Amplicon size ranged from 70 to 88 base pairs and the number of alleles observed was 4. This genetic variability proved to be enough to identify the cultivars involved in each cross. In this limited study, we found that the cultivar ‘Okei’ pollinized twice as many nuts on ‘Colossal’ as did the cultivar ‘Benton Harbor’. Results from this study provide essential information regarding the genetic characterization of chestnut cultivars and the importance of conducting controlled pollination experiments to understand its effect on nut production.

INTRODUCTION

Chestnuts (*Castanea* spp.) are native to the Appalachian forest of North America and were a co-dominant species until the accidental introduction of the fungus *Cryphonectria parasitica*, the causal agent of chestnut blight. In an effort to replace the endemic American chestnut (*C. denata*) with a chestnut blight resistant chestnut tree, Japanese and Chinese chestnut trees (*C. mollissima* and *C. crenata*, respectively) were introduced. Today, the Michigan population of chestnut trees consists of remnant populations of American chestnut trees, and both seedling and grafted cultivars of Chinese, Japanese and European chestnut trees and their hybrids (Fulbright et al, 2010). These trees, including the American chestnut, were mainly planted as orchard trees for nut production. In the late 20th century and early 21st century, thousands of cultivars, (*e.g.* 'Colossal', a European x Japanese hybrid), were planted in Michigan chestnut orchards to produce orchards with high yields and nuts with marketable sizes (Fulbright and Mandujano 1999). Therefore, since the introduction of chestnut blight in the early 20th century, the diversity of chestnut germplasm has increased primarily due to chestnut blight and the goal of growing high yielding chestnuts. In an effort to increase the chestnut production in Michigan, unfortunately a large number of the cultivars introduced to the state have uncertain provenance and/or the grafted trees have inconsistent traits to be considered true-to-type representatives of the cultivar.

Recognizing that all *Castanea* species and cultivars can hybridize (i.e. cross-pollinate), the identification of the chestnut genotypes and cultivars is complex and challenging. While older references suggest that chestnut species can be distinguished by traits such as leaf hairs, leaf shape, nut size and nut pubescence for species identification (Graves 1992), the reality is

that these traits are much too variable (Miller 2003) especially when hybrid and cultivar determination is required.

In the 1980s, chestnut trees used to establish orchards in the Midwest including Michigan were established using chestnut seedlings with mixed genetic backgrounds, some known cultivars grafted on mixed seedling rootstocks by in-state or out-of-state nurseries, and selections of genetically unknown trees grafted onto mixed rootstock by grower orchardists. Most growers identify trees in their orchards based on the species it most likely resembles (such as Chinese chestnut), and/or by cultivar name if purchased as grafted nursery stock.

Growers are dependent on nurseries for correct identification of scion wood and grafted trees. If the graft dies, the tree may continue to live as rootstock with sucker sprouts of unknown heritage knowingly or unknowingly replacing the cultivar. In addition, many growers believe they are growing Korean chestnut seedling trees or Manchurian chestnut seedling trees, names that have no valid species designation.

Instituting orchards with cultivars of known genetic parentage is important if markets are or to become sophisticated enough to demand certain traits in the products. Seedling trees producing variable types of chestnuts may be adequate for some types of markets, but when standards are required, such as by grocery store chains, certain traits are expected such as size, color, taste, and shelf life.

Establishing chestnut orchards with high quality, grafted cultivars has been problematic in Michigan for several reasons. Growers are still told that seedlings trees are adequate for the markets available, seedling trees are more economical and common, and seed from the growers' orchards is readily available to start new trees. Grafted trees with a good reputation for growth and production, on-the-other-hand, are at one time unknown, production trees are relatively

expensive, and are not commonly available, their reputation, whether deserved or not, would frequently die after planting due to graft incompatibility.

While there has been progress in the past decade with growers establishing orchards with known, reputable, grafted cultivars, the older seedling-based orchards still exist and are still being established due to word of mouth dealings between growers. In seedling orchards, growers may call seedling trees by their last known maternal tree. For example, it is not uncommon to hear that certain trees in a seedling orchard are 'Colossal' seedlings, sometimes truncated to 'Colossal'. The former designation may be accurate, in that the seed that produced the current trees were from a grafted 'Colossal' mother tree, but it does not take into consideration the paternal tree that pollinized the 'Colossal' tree. Other times growers may believe they have Chinese chestnut seedlings, when they actually have seedlings from a Chinese mother tree pollinized by an unknown paternal tree. If that paternal tree was an American chestnut tree growing nearby, the resulting "Chinese chestnut trees" (actually hybrids) would likely be chestnut blight susceptible instead of resistant as expected for pure Chinese chestnut. The outcome of this is an industry based on irreproducible, inadequate germplasm, without the ability to obtain desired traits and reproduce those traits in new orchards. It is often observed that the favorable traits of the trees selected by the growers at first are lost in time, and the non-favorable traits of the trees may become accentuated due to either recombination at fertilization, or the expression of "new" previously unknown traits, or both that can only bee observed on the new seedling tree (*i.e*. progeny). Therefore, cultivars must be found with the traits desired by growers and the market. These traits must be reproduced with clonal germplasm, where the clonal germplasm must be made readily available and the grafted or budded trees must be transplantable with a high degree of success.

Michigan and other states in the Midwest are leaders in the nascent chestnut industry when measuring acreage and number of growers (*The Census of Agriculture*, 2007). As stated above, many of the orchards in Michigan are based on seedling trees, however, in the past decade newly established Michigan chestnut orchards have been initiated with cultivars carrying traits important to the industry, such as size of nut, yield, taste and other important sensory qualities. The growers are largely dependent on nurseries, university researchers, or friends for providing grafted trees or scion wood. However, what ends up in the orchard may be something different than what the grower thought due to the common problems of improperly labeled germplasm, propagation of rootstock instead of cultivar germplasm due to loss of the graft and subsequent growth of sucker sprouts mimicking the desired cultivar, and inevitable accidents of mislabeling trees, which may occur even during the best of horticultural practices.

Due to the variability of chestnut, its ability to hybridize with other species, and potential mistakes made during propagation of known cultivars, the only real answer to policing germplasm is with genetic markers, such as simple sequence repeats (SSRs), also called microsatellites. These markers have been widely used as a reliable method for the identification of varieties and cultivars of many crops; from perennial and annual crop plants, to flowering and non-flowering trees (Powell et al, 1996). However, the use of SSRs as genetic markers of chestnuts in North America has not been extensively explored in part due to the lack of a commercial industry. Recently, the American Chestnut Foundation has employed SSR technology to determine population structure of *Castanea* in North America (Kubisiak and Roberds 2006) and the genetic mapping of chestnut blight resistance genes in Chinese and American chestnut species (Sisco et al, 2005; Barakat et al, 2009). Recently, McCleary et al. (2013) conducted an extensive study using 11 expressed sequence tag (EST) SSR, as the genetic

markers of choice to clarify the ancestry of chestnut cultivars that had conflicting pedigrees due inconsistent cultivar's name records due to mislabeling or unknown interspecific pedigrees. In their study, McCleary et al (2013) were able to correct or establish the interspecific pedigree of 65 chestnut cultivars, which included hybrids, seedlings and representative trees of Chinese (*C. mollissima*), European (*C. sativa*), Japanese (*C. crenata*), American (*C. dentata*) and *C. pumila* species. Furthermore, McCleary et al (2013) is the first report on the use of EST-SSR as a tool to genetically identify synonymy and homonymy on cultivars currently grown in the United States.

The objectives of this study were to identify chestnut cultivars currently growing in Michigan using SSRs as genetic markers, and to determine the efficacy of SSRs in the identification of cultivars used in a tri-parental cross and its progeny. Results for this study will be considered as the primary steps for the establishment of a program for the identification and validation of various hybrids, and species. This could play an important role in helping the industry establish genetic standards. Based on preliminary data reported by other scientists and myself (Medina-Mora 1999; Buck et al, 2003; Marinoni et al, 2003), I hypothesized that three to five SSR markers will be necessary to identify the chestnut cultivars currently growing in Michigan, and that two to five SSR markers will be sufficient to perform paternity analysis. The data will be collected and analyzed until enough trees of each cultivar from various locations are included in the study, including the original tree used to start the grafted clone tree (*i.e.* ramet) also known as ortet (if available). The data collected for each cultivar will be limited by the resources available to perform the study, as there is no procedure to determine which is the correct DNA fingerprint for a specific cultivar other than provenance.

MATERIALS AND METHODS

SSR-based Identification of Chestnut Cultivars

To determine the usefulness of SSR primers as genetic markers of 11 chestnut cultivars currently growing in Michigan orchards were genotyped.

Plant Material

A total of 113 chestnut leaf or bark samples representing a total of 8 European x Japanese hybrids (*C. sativa* x *C. crenata*) and Chinese (*C. mollissima*) chestnut cultivars commonly grown in Michigan were used to evaluate previously described simple sequence repeats (SSRs) markers for *Castanea* species. Fifty-eight of the 113 samples collected representing 5 European x Japanese hybrid cultivars including 'Bouche de Betizac', 'Colossal', 'Mariguole', 'Nevada', and 'Precoce Migoule' (Table 1). Thirty-four samples of 'Colossal' were included in this study because 'Colossal' is the predominant cultivar in Michigan orchards due to the large number of plantings that occurred during late 1990's and early 2000's. All samples of 'Colossal' were collected from 2 private chestnut orchards, 1 nursery, and 3 Michigan State University (MSU) research plots located in East Lansing (Ingham County), Clarksville (Ionia County), and Benton Harbor (Berrien County), Michigan. Two samples of 'Nevada' were included because this cultivar was introduced to Michigan orchards as a pollinizer of 'Colossal' due to its synchrony with 'Colossal' flowers. Samples of 'Mariguole' and 'Precoce Miguole' were included because it is thought that these two cultivars have been improperly labeled due to possible mistakes that could have occurred during propagation. Genotyping 'Mariguole' and 'Precoce Migoule' could potentially be a good example of synonymy. Samples of 'Bouche de Betizac', 'Precose Miguole' and 'Marigoule' were included in this study because these cultivars are considered French hybrid chestnuts (*C. sativa* x *C. crenata*) and they are currently cultivated

Table 1. Name of cultivars, putative pedigree, number of samples per cultivar, origin, and source of samples included in this study.

Hybrid	Pedigree (putative)	Cultivar	Number of samples	Origin [a]	Source [b]
European—Japanese	*C. sativa* X *C. crenata*	'Colossal'	34	CA	MI
		'Nevada'	2	CA	MI
		'Marigoule'	3	France	MI
		'Precose Migoule'	13	France	MI
		'Bouche de Betizac'	6	France	MI
Chinese	*C. mollissima*	'Benton Harbor'	25	FL	MI
		'Everfresh'	5	MI	MI
Chinese— (Japanese— American)	*C. mollissima* X (*C. crenata* X *C.dentata*)	'Eaton'	12 [c]	CT	MI (9)
				CT	CT (3)
		'Eaton River'	1	KY	MI

a Place where tree begins to develop or where cross takes places; CA=California, USA, FL=Florida, USA, MI= Michigan, USA, CT=Connecticut, USA, and KY=Kentucky, USA.

b Physical location where tree is currently growing and from where sample was collected; MI=Michigan, USA, and CT= Connecticut, USA.

c 9 of the 12 samples were collected from trees currently growing in Michigan, while 3 of the 12 samples were collected from trees currently growing in Connecticut.

Table 1. (cont'd)

Hybrid	Pedigree (putative)	Cultivar	Number of samples	Origin [a]	Source [b]
Japanese—Allegheny chinquapin	*C. crenata* X *C. pumila* var. *pumila*	'Okei'	2	CA	MI
Japanese—Chinese ? (Korean)	*C. crenata* X *C.mollissima*	'J160'	6	MI	MI

in Michigan. Thirty-five of 113 samples collected represented two cultivars, 'Benton Harbor', and 'Everfresh', thought to be selections from open-pollinated Chinese seedling trees (Table 1). Both cultivars have been grafted to unknown genetically mixed Chinese rootstock and distributed among chestnut growers in Michigan. From the samples included in this study for these two Chinese selections, 5 leaf samples were collected from the ortet of 'Benton Harbor', and one leaf sample was collected from the ortet of 'Everfresh'. The remaining samples from 'Benton Harbor' and two samples of 'Everfresh' were collected from ramets growing at MSU research stations in Clarksville and Benton Harbor, Michigan. The remaining two samples of 'Everfresh' were collected from seedling trees. Additionally, two samples of cultivar 'Okei' were included in this study because this cultivar was also introduced to Michigan orchards following recommendations by the nursery that grafted and distributed 'Colossal' and 'Nevada'. A total of 9 samples were collected from ramets of 'Eaton' (Table 1). 'Eaton' is thought to be a three-way cross hybrid (Anagnostakis 1999), where one parent is the F1 hybrid between Japanese x American chestnut species (*C. crenata* x *C. dentata*) and the other parent is Chinese chestnut species (*C. mollissima*). Three chestnut samples considered to be 'Eaton' ortets were included in the study to compare the SSR profiles of these 'Eaton' ortets with 'Eaton' cultivars currently growing in Michigan. 'Eaton' ortets are located in Connecticut and leaf samples were kindly provided by Dr. Sandra Anagnostakis (The Connecticut Agricultural Experiment Station). In addition, one sample of a cultivar named 'Eaton River' was included in the study because grafted trees of 'Eaton River' were thought to be the same as 'Eaton', however Dr. Anagnostakis (*personal communication*) thought these cultivars were genetically different due to morphological dissimilarity. Hence, the cultivar named 'Eaton River' represents grafted trees propagated and distributed by Nolin River Nut Tree Nursery, Kentucky (www.nolinnursery.com)

and these trees are not the same as the ramets from the 'Eaton' growing in Connecticut. Analyzing the SSR profiles of these two cultivars could be an example of SSR markers' usefulness in the identification of synonyms and homonyms. Furthermore, a total of 6 samples of the cultivar known as ‘J160’ was included in this study because it is thought that this tree could be the source of pollen for ‘Colossal’ female flowers early during the pollination time of ‘Colossal’ trees growing in Michigan orchards.

To test reproducibility of SSR-based genotyping, four 'Colossal' leaf samples were collected from branches pointing to each of four cardinal points (north, south, east, and west) (Table 1). In addition, four 'Benton Harbor' bark samples were collected from branches of the 'Benton Harbor' ortet at SWMREC. These four samples represented 'Benton Harbor' branches extending from the main truck to cardinal points (north, south, east, and west). These four samples were excluded from the phenogram construction included in this study (see below).

Because buds from unknown rootstock may grow in the event of graft failure, one leaf sample from sprouts growing at the base of four 'Colossal' trees were included in this study to test if rootstock of theoretically close, but unknown pedigree could have different SSR profiles from 'Colossal' grafted trees (Table 1).

DNA Extraction

DNA was extracted from 0.05 g of leaves or bark using a modified procedure described by Hamelin et al. (2000), and manufacturer’s recommendations by QIAGEN (DNeasy Plant Extraction Kit, Qiagen Sciences, Massachusetts, USA) in the substitution of QIAGEN Extraction buffer with 2% CTAB buffer without β-mercaptoethanol. After elution, each DNA sample was quantified and its concentration calculated using NanoDrop 3300 Fluorospectrometer (Thermo Scientific, Wilmington, Delaware, USA). For each sample, an aliquot of 50 ng/μL (final

concentration) was suspended in double-distilled H_2O and stored at -20°C. DNA of every sample was extracted from the same leaf or bark tissues two times, and the remaining tissues were stored at -20°C. Aliquots of each sample were thawed on ice for approximately 30 min. prior to PCR amplification.

Polymerase Chain Reaction (PCR) Amplification

A total of five SSR loci previously described by Buck et al. (2003) and Marinoni et al. (2003), were evaluated in this study including EMCs15 (Buck et al, 2003), CsCAT1, CsCAT2, and CsCAT16 (Marinoni et al, 2003), QrZAG75 (Kampfer et al, 1998; Boccacci et al, 2004) (Table 2). Because 'Colossal', the most predominant cultivar grown Michigan is a European x Japanese hybrid (*C. sativa* x *C. crenata*), the EMCs and CsCAT primer sets were selected to be evaluated in this study because as these primers were designed for genotyping European chestnut species (*C. sativa*), I hypothesize that these primers would also amplify 'Colossal'. Furthermore, several CsCAT primer sets have been previously tested to hybridize with genomic DNA extracted from chestnuts trees known to be Chinese x American hybrids (*personal communication*, Paul Sisco and Thomas Kubisiak, The American Chestnut Foundation). The primer pair for each SSR locus was included with the following reagents in the PCR into the PCR master mix; 1X AFLP amplification core mix (Applied Biosystems-ABI, Life Technologies Corporation, Carlsbad, California, USA), 0.5μM of each primer, 0.2 U Ampli-*Taq* Gold polymerase (ABI, California, USA) and 50 ng of template DNA, for a final volume of 20 μl. Amplification cycles were performed according to Marinoni et al. (2003) following the suggested annealing temperature specific for each primer pair. However, the annealing temperature for each primer pair was modified to avoid the amplification of DNA fragments (*i.e.*

Table 2. Simple sequence repeats (SSR) primers used for the analysis in this study, annealing temperature, motif and size of alleles for each primer pair previously published.

SSR locus name [a]	Primer sequence [b]	Annealing temp [c] (degree C)	Motif [d]	Allele size (bp) [e]		Reference [f]
				predicted	observed	
CsCAT1	F: GAGGAATGCCCACTTTTGCA R: GCTCCCTTATGGTCTCG	50	$(TG)_5TA(TG)_{24}$	220	166 - 222	Marinoni et al. 2003
CsCAT2	F: GTAACTTGAAGCAGTGTGAAC R: CGCATCATAGTGAGTGACAG	55	$(AG)_{16}$	206	173 - 216	Marinoni et al. 2003
CsCAT16	F: CTCCTTGACTTTGAAGTTGC R: CTGATCGAGAGTAATAAAG	50	$(TC)_{20}$	143	120 - 171	Marinoni et al. 2003
EMCs15	F: CTCTTAGACTCCTTCGCCAATC R:CAGAATCAAAGAAGAGAAAGGTC	52	$(CAC)_9$	83 - 95	70 - 90	Buck et al. 2003

a CsCAT= *Castanea sativa* Colture Arboree Torino; EMCs=East Malling UK, *Castanea sativa*; QrZAG=*Quercus robur*, Zentrum fuer Angewandte Genetik, Vienna

b F=forward primer labeled with fluorescent dye (6-FAM, HEX, or NED), R=reverse primer

c Annealing temperature for each primer pair previously reported by researchers (see reference below)

d Repetitive motif of SSR locus

e Reported and observed (in this study) size or size range for alleles in basepairs (bp)

f Marinoni et al. 2003. Mol. Breed. 11:127-136; Buck et al. 2003. Mol. Ecol. Notes 3:239-241; Kampfer et al. 1998. Hereditas 129:183-186; and Boccacci et al. 2004. HortSci. 39:1212-1216

Table 2. (cont'd)

SSR locus name [a]	Primer sequence [b]	Annealing temp [c] (degree C)	Motif [d]	Allele size (bp) [e]		Reference [f]
				predicted	observed	
QrZAG75	F: ACCGCCTATCTCAACCAGAG R: GTCCGAGAATCATCATTAAAGG	58	$(GA)_{57}$	116 - 182 112 - 166	98 - 168	Kampfer et al. 1998 Boccacci et al. 2004

amplicons) of unexpected band size. The forward primer of each primer pair was labeled with a fluorochrome (6-FAM, HEX, or NED) necessary for automatic analysis of amplicons. Each template DNA was amplified six times and the amplicons combined into one sterile microcentrifuge tube per sample. Amplicons were resolved on 1% agarose gels stained with ethidium bromide. One hundred fifteen μl of the amplicons were purified using QIAquick PCR purification columns (Qiagen Sciences), and suspended in 30 μL double-distilled water and stored at -20°C. Two to three hundred μls of the re-suspended amplicons were resolved using a capillary-based electrophoresis, ABI PRISM 3130 Genetic Analyzer (Research Technology Support Facility-Genomics Core, East Lansing, Michigan). All leaf samples were subjected to three independent series of PCR amplification prior to microsatellite analysis, however, bark samples were only amplified one time because of the limited amount of DNA extracted from the bark.

Evaluation of Polymorphisms

In order to evaluate the SSR marker polymorphism, we used GeneScan Analysis software (Perkin Elmer, Massachusetts, USA) to automatically identify all amplicons, compare the mobility of each amplicon to that of the internal lane standards (GS500), and size it based on the sizing curve of the internal lane standards. The length of each amplicon was determined using computer software such as Genotyper v.3.7 (ABI Prism, California, USA) and Peak Scanner v.1.0 (ABI, California, USA).

One DNA sample form each cultivar was randomly selected to evaluate the ability of each SSR locus to generate amplicons informative to our study. An informative SSR marker was described as a co-dominant and polymorphic locus for all 11 cultivars. And, a non-informative marker was described as an SSR marker where more than two alleles were observed (*i.e.* non-

specific) for an individual, or if the SSR marker was a monomorphic locus for all 11 cultivars. Further analysis of the microsatellite data was conducted for all DNA samples included in this study if the SSR locus was informative.

Microsatellite Analysis

The efficiency of each SSR locus to differentiate chestnut cultivars was determined by analyzing the allelic frequencies, the expected and observed heterozygosity, the frequency of null alleles, the polymorphic information content (PIC), the power of discrimination (PD), the paternity exclusion probability (PE), and the probability of identity (PI). All of these parameters were calculated using one of three computer programs; IDENTITY 4.0 (Wagner and Sefc 1999), POPGENE 1.31 (Yeh et al, 1999), or CERVUS 3.0 (Field Genetics Ltd. London, UK; Kalinowski et al, 2007). The number of alleles, the allele frequency per locus, and the observed heterozygosity (Ho) were obtained from direct calculations of the SSR data. The number of effective alleles (ne) was calculated using POPGENE software following the equation described by Kimura and Crow (1964). The effective number of alleles (ne) for a diploid population was calculated as ne = 1/ F where F is the probability that an individual would be homozygous. The effective number of alleles (ne) of a SSR locus is a parameter used to describe the level of heterozygosity observed at each locus, since ne is the inverse of F. When the effective number of alleles (Kimura and Crow's equation) is applied to molecular data as SSR, it can be defined as "the number of equally frequent alleles that would produce the same heterozygosity observed [per SSR locus]" (McDonald 2008). The expected heterozygosity (He) was calculated as He = $1- \sum p_i^2$ (Nei 1973) where p_i denotes the frequency of allele i in the individuals. The frequency of the null alleles was calculated as r = (He-Ho)/(1 + He) following Brookfield (1996), where He = expected heterozygosity and Ho = observed heterozygosity. The polymorphic information

content (PIC) is the probability that the genotype of the offspring of a heterozygous parent including a dominant trait allows one to assume which allele the offspring inherited from the parent. PIC is considered a measurement of marker's usefulness for linkage analysis regardless of the model of inheritance of the alleles (Guo and Elston 1999). PIC values > than 0.5 indicates that a locus is highly informative, while PIC values < than 0.5 specifies that a locus is slightly informative. The power of discrimination was calculated for each SSR locus as $PD = 1- \sum g_i^2$, where g_i is the frequency of the allele i (Jones 1972). PD is an estimate of the probability that two random genotypes could be distinguished by their SSR profiles. The paternity exclusion probability (PE) was calculated following Weir (1996) and the probability of identity was calculated as $PI = 1- p_i^4 + (2p_i p_j)^2$ (Paetkau et al, 1995), where p_i and p_j = frequency of alleles i and j, respectively. PE refers to the probability of excluding a random individual as a potential parent of the individual based on the genotype of one parent and the offspring. PI is the probability of two unrelated individuals showing the same multilocus genotype by chance. The total PE and total PI for all 5 SSR loci included in this study were calculated by the sequential products of the PE and PI values obtained for each locus. Identical genotypes among the samples included in this study were identified using the computer software IDENTITY, where identical genotypes were assigned only to samples that resulted of identical SSR profiles across all 5 SSR loci.

Phenogram Construction

A phenogram of the 110 chestnut cultivars/hybrids, excluding the SSR profile obtained for the four bark 'Benton Harbor' ortet samples, was constructed using the UPGMA (unweighted pair-group method with arithmetic averages) based on the genetic distance between population calculated using Nei's (1972) formula: $D = 1–\log_e I$, where I represents the proportion of identical

alleles between two samples. The phenogram was constructed considering all individuals genotyped as homozygotes at each SSR locus as if the diploid individual had two copies of the same allele and the same allele size (*i.e.* genotype 124, 124). The Tree plot module of the NTSYSpc 2.20v N software (Rohlf 1998; www.exetersoftware.com) was used to visualize the phenogram.

SSR-based Evaluation of Progeny from an Open-pollinated Orchard in Michigan

To test the efficacy of SSR primers as genetic markers paternity analysis was conducted for the paternal trees and the F1 progeny of a tri-parental cross.

Chestnut Orchard and Pollen Source

An 8-years old chestnut commercial orchard located in Eaton Rapids, MI (Eaton County) was selected as the study site because the limited number of chestnut cultivars planted, which allowed an easier site to monitor pollination and evade the complexity of other orchards with multiple cultivars and seedlings. The orchard consisted of two single rows of trees following a 7.5 x 7.5 m design, where one row consisted of four trees of the European x Japanese (*C. sativa* x *C. crenata*) cultivar 'Colossal'. The European x Japanese cv 'Colossal' trees planted at this orchard in 2002 as grafted trees originated from Fowler Nursery, Newcastle, California (Nowadays, no longer propagating and selling chestnut trees). 'Colossal' has been the unofficial leader in chestnut production in Michigan not only because of the large number of trees planted in the late 1990s and early 2000s, but also because of its yield advantages (Fulbright and Mandujano 1999), and the favorable characteristics of the nuts such as flavor (Kelley and Behe, 2002; Warmund et al, 2011) and size (Bassi and Craddock 1998; Fulbright 2011). 'Colossal', like all chestnuts, is monoecius and self-incompatible, and also is male-sterile, thus female 'Colossal' flowers may be pollinized after out-crossing. Nut-set may take place after receptive

female flowers receive airborne pollen from another chestnut tree (*i.e.* pollinizer) (Ohata and Sato 1961). The second row of trees in the orchard consisted of two trees, which served as pollen source or pollinizers of 'Colossal' female flowers. A single Japanese x Allegheny chinquapin (*C. crenata* x *C. pumila* var. *pumila*) tree, 10 m in height, was planted in 2002 during the same time as all four 'Colossal' trees. The 'Okei' (Anagnostakis 1999) grafted tree was also originally propagated and distributed by Fowler Nursery, and it was sold as the best pollinizer tree for 'Colossal' because according to Fowler's Nursery 'Okei' pollen dehiscence time synchronized with the receptive period of 'Colossal' female flowers. A single Chinese selection 'Benton Harbor' (*C. mollissima* seedling cv. 'Dunstan Hybrid') grafted tree, 4 m in height, was planted in 2005 at this orchard as an alternative pollinizer for 'Colossal', since the 'Okei' tree has been reported to be affected by the erratic cold temperatures Michigan experience during early Spring. 'Benton Harbor' propagated and distributed by Michigan State University personnel, seems to have been well adapted to withstand Michigan's cold temperatures, and the tree was selected due to its copious production of pollen, large yields of high quality nuts in spite of been a Chinese chestnut tree (Fulbright and Mandujano 1999; Fulbright 2011).

Pollination

On 20-June 2010, a total of 120 'Colossal' female flowers were covered with pollination bags (Lawson #451, Illinois, USA) before pollen dehisce from the 'Benton Harbor' and 'Okei' trees. Receptive 'Colossal' female flowers consisted of one central group of styles approximately 2 mm longer than the 2 lateral style groups. On 23-June 2010, 60 flowers were uncovered and exposed to airborne pollen. On 10-July, these 60 flowers were covered again when approximately half of the male catkins on the 'Benton Harbor' tree were shedding pollen. The remaining 60 'Colossal' female flowers remained covered until 12-July, when flowers were

uncovered and exposed to airborne pollen. As a control, a total of 60 flowers remained uncovered and exposed to pollen during the entire blooming period. In late-August, all 120 flowers were covered with mesh bags (PB-507, Monte Package Corporation, Michigan, USA) to aid in the collection of burs and nuts at harvest. By this time, catkins of 'Benton Harbor' and 'Okei' trees were dry and brown in color, an indication that pollen dehiscence had ceased.

Chestnut burs were harvested on 22-September and stored at 4°C until nuts were collected on 30-September. The total number of burs, the number of nuts, the position of nut within each bur, and the weight of each nut were recorded. Each nut was surface sterilized using 75% ethanol and allowed to air dry. To track each nut (*i.e*. F1 progeny) from seed to seedling, every nut was randomly enumerated using a permanent maker on the helium scar (natural spot).

Plant Material and DNA Extraction (Kernel Biopsy)

One-to-two young leaves were collected from all parental cultivars ('Colossal', 'Benton Harbor', and 'Okei') and stored at -20°C in plastic bags until DNA extraction (described below). From each nut, approximately 0.02 g of kernel tissue was extracted from each numbered nut using a sterile bone marrow biopsy instrument (13 gauge, 5 cm, CareFusion Corporation, California, USA). The bone marrow biopsy needle was strongly but gently introduced through one side of the shell of the nut into the kernel tissue, carefully preventing the needle from perforating the opposite side of the shell. Each kernel biopsy sample was deposited inside a sterile mortar and pestle and the DNA from the sample was immediately extracted (Figure 1). Between each sample, the bone marrow biopsy needle was surface sterilized by exposing the needle tip and the barrel to the flame of a laboratory gas burner, and allowed to cool down between samples. The hole created within the kernel tissue and the shell was filled with silicone

(Translucent GE 1200 sealant, North Carolina, USA), allowed to cure for 10 min and stored at 4°C for stratification (Figure 2). After 100 days of stratification, nuts were removed from refrigeration and visually analyzed for nut quality. Each nut was planted in Baccto potting soil (Premium, Michigan peat company, Huston, Texas, USA) and allowed to germinate into a seedling under greenhouse conditions. Seedlings were kept in the greenhouse until the end of this study in order to harvest leaf samples of each seedling for future experiments. DNA was extracted from 0.02 g of kernel tissue using a modified procedure as described above.

Polymerase Chain Reaction (PCR) Amplification

To determine the SSR genotype of the parental cultivars ('Colossal', 'Benton Harbor', and 'Okei') and the F1 progeny, two SSR loci, previously described by Buck et al (2003) and Marinoni et al (2003), were used in this study. SSR loci EMCs15 (Buck et al, 2003; Medina and Fulbright 2010) was used to genotype all 70 nuts, while CsCAT1 (Marinoni et al, 2003; Medina and Fulbright 2010) was used to verify the genotype of 15 nuts with ambiguous genotypes.

The DNA of each sample was subjected to a minimum of two independent PCR amplification runs. One DNA extraction was performed from all kernel tissue samples, and subjected to a single PCR run as indicated above. A second DNA extraction and PCR run were performed for each F1 progeny (*i.e.* kernel tissue sample) using a leaf sample from the seedling that germinated under greenhouse conditions.

Simple Sequence Repeats (SSR)-based Analysis of Parents and F1 progeny

To assign a SSR genotype to the parental cultivars and the F1 progeny, GeneScan Analysis software (Perkin Elmer, Massachusetts, USA) was used to automatically identify all

Figure 1. Kernel tissue from nuts was extracted from each nut using a sterile bone marrow needle (13G X 2 inches). Approximately 0.02 grams of tissue was collected from each nut, and DNA was successfully isolated from each kernel's tissue sample, and subjected to SSR-genotyping.

Figure 2. The hole created by the bone marrow biopsy needle on the surface of the shell was sealed using silicone, which replaced the empty space in the kernel created by the biopsy needle. The silicone in each nut was allowed to cure for 10 min before nuts were stored at 4°C for stratification.

amplicons and compare their mobility to that of the internal lane standards (GS500) and size based on the sizing curve of the internal lane standards. The length of each amplicon (i.e., allele size of SSR locus) was determined using computer software, Genotyper v.3.7 (ABI, California, USA) and Peak Scanner v.1.0 (ABI, California, USA). The assignment of the SSR profiles for each parental cultivar and the F1 progeny (*i.e.* nuts) were conducted visually upon comparison of the SSR alleles observed for each SSR locus, and each SSR allele size was reported in basepairs (bp) (Table 3).

The efficacy of each SSR locus to differentiate the paternal cultivars and the F1 progeny was determined by analyzing the expected and observed heterozygosity, the polymorphic information content (PIC), the paternity exclusion probability (PE), and the probability of identity (PI). The statistical formulas for these parameters used were the same as the formulas described above.

RESULTS

SSR-based Identification of Chestnut Cultivars

To evaluate SSR markers developed for European chestnut species (*Castanea sativa*) and oak species (*Quercus robur*), 114 samples representing the 11 chestnut cultivars included in this study were amplified following PCR amplification methodology as previously described (Buck et al, 2003; Marinoni et al, 2003; Kamper et al, 1998).

SSR locus Analysis

Successful PCR amplification and unambiguous amplicons were obtained with 5 SSR primer pairs; CsCAT1, CsCAT2, CsCAT16, EMCs15 and QrZAG75. Primer pairs, CsCAT1, CsCAT2, and CsCAT16 yielded amplicons within the previously reported size range (Table 2) but, primer pairs EMCs15 and QrZAG75 yielded amplicons of smaller size than

Table 3. Chestnut cultivars/hybrids and flower type of cultivars used as paternal trees on this study, and SSR alleles (basepairs) expected per SSR locus for the parents and its progeny.

Cultivar parent	**Hybrid**	**Flower type**	**SSR locus** allele size (base pairs)		**Expected SSR genotypes of F1 progeny**			
					EMCs15		**CsCAT1**	
			EMCs15 [a]	**CsCAT1** [b]	'BH'	'Okei'	'BH'	'Okei'
'Colossal'	European X Japanese	female	76, 88	183, 191	70,76	76,85	176,183	183,191
					70,88	85,88	176,191	183,— [c]
'Benton Harbor'	Chinese selection	pollen	70, 76	176, 184	**76,88** [d]	**76,88**	183,184	191,—
'Okei'	Japanese X Chinquapin	pollen	85, 88	183, 191	76,—	88,—	184,191	

a EMCs = East Malling UK, *Castanea sativa* (Buck et al., 2003)

b CsCAT = *Castanea sativa* Colture Arboree Torino (Marinoni et al. 2003)

c — represents a homozygous genotype, where an individual carries two alleles of the same size (base pairs).

d The presence of the 76 bp and 88 bp at the EMCs15 locus, conferring an heterozygous genotype to a nut causes ambiguity as to which one of the two alleles was inherited by the 'Benton Harbor' or the 'Okei' pollen donor.

previously reported by Buck et al (2003), and Boccacci et al (2004), respectively.

SSR loci Polymorphism

All 5 SSR loci selected for fingerprinting chestnut cultivars and hybrids included in this study were polymophic, where more than two alleles were observed among the 110 leaf samples. The number of alleles, the number of effective alleles, the observed and expected heterozygos polymorphic information content (PIC), power of discrimination (PD), probability of exclusion (PE), and probability of identity (PI) were used to evaluate polymorphism of each locus (Table 4).

The number of alleles per locus ranged from 10 to 19 (Table 4). Loci CsCAT2 and CsCAT16 (19 alleles each locus) had the largest number of alleles. The range of the effective number of alleles (ne) was 3.4 to 5.4, while the CsCAT16 locus was found to contain the lowest effective number of alleles and QrZAG75 locus consisted of the highest effective number of alleles. The values of observed heterozygosity (Ho) and expected heterozygosity (He) across all loci ranged from 0.457 to 0.923 and 0.711 to 0.824, respectively. All Ho values were higher than the He values, except for the Ho obtained for CsCAT16 locus. A higher value of Ho than He is considered as a clear indicator of the high level of genetic diversity across all loci, with the exception of CsCAT16. The excess of homozygosity observed at the CsCAT16 locus is in congruency with the positive number obtained for the frequency of null alleles (+0.245) at this locus, and the lowest number of effective alleles (3.4) observed for this data set (Table 4). A positive value for the frequency of null alleles implies the exclusion of CsCAT16 primer pairs from future parenting analysis but not from the use of this locus for genotyping. In addition, a lower value of Ho than He indicates a higher number of homozygotes than expected, thus it

Table 4. Characteristics of the 5 SSR loci assessed in the 110 chestnut cultivars/hybrids included in this study.

SSR locus name [a]	Number of			Frequency of [e]			PIC [f]	PD [g]
	samples [b]	alleles $_{obs}$ [c]	ne [d]	Ho	He	null		
CsCAT1	94	17	5.3	0.862	0.810	-0.034	0.783	0.807
CsCAT2	98	19	4.0	0.755	0.751	-0.035	0.724	0.747
CsCAT16	94	19	3.4	0.457	0.711	+0.245	0.693	0.707
EMCs15	97	12	5.2	0.866	0.814	-0.024	0.79	0.811
QrZAG75	91	10	5.4	0.923	0.824	-0.065	0.797	0.819

a CsCAT= *Castanea sativa* Colture Arboree Torino; EMCs=East Malling UK, *Castanea sativa*; QrZAG=*Quercus robur*, Zentrum fuer Angewandte Genetik, Vienna

b Number of samples included in the analysis differ from the total number of samples because a limited number of samples were not typed with every locus included in this study.

c Number for alleles observed from direct counting of alleles in this study.

d Effective number of alleles calculated using POPGENE software following equation described by Kimura and Crow (1964).

e Frequency of heterozygosity: Ho= observed frequency of heterozygotes, and He= expected frequency of heterozygotes for this data set following Nei's (1973). Null= estimated the frequency of null alleles calculated using CERVUS software according to (Summers and Amos 1997).

f Polymorphic information content (PIC) calculated using CERVUS software according to Kalinowski et al, 2007.

g Power of discrimination (PD) following formula described by Jones (1972).

represents a lower level of genetic diversity at the CsCAT16 locus, which suggests a certain level of inbreeding.

In general, every SSR locus included in this study is highly informative because PIC and PD values were higher than 0.5. The PIC and PD ranged from 0.693 to 0.797 and 0.707 to 0.819, respectively (Table 4). The highest PIC and PD values were observed in QrZAG75 locus. The lowest PIC and PD values were obtained for the CsCAT16 locus, as expected from the observation regarding the frequency of null alleles. Regardless of the low values observed with CsCAT16, all 5 loci are informative and useful for genotyping the 114 samples included in this study.

The probability of exclusion (PE) for each locus ranged from 0.554 for CsCAT2 to 0.645 for EMCs15 and QrZAG75, where the total probability of exclusion was 0.991. Higher values than 0.5 for PE demonstrates the usefulness of these 5 SSR loci for future studies on parentage verification. The probability of identity (PI) for each locus ranged from 0.056 for EMCs15 and QrZAG75 to 0.096 for CsCAT16, where the total probability of identity was 1.61×10^{-6}. In general, rates lower than 1×10^{-4} for total PI are required for unambiguous identification of individuals. Thus, unambiguous identification of the 114 samples included in this study could be obtained when the genotype at each SSR locus is combined to generate a unique SSR profile for each sample.

Overall, the allelic frequency per locus observed in these data sets ranged from 0.005 to 0.516, where the 124 bp allele of CsCAT16 was found to be the most frequent allele of the entire data set (Table 5). As expected, when the frequency of alleles for each SSR locus was analyzed independently a range of alleles frequency could be described for each SSR locus. The 184 bp

allele was the most frequent allele for CsCAT1 locus, while the 166 bp, 181 bp, 189 bp, and 195 bp alleles were the least frequent alleles. The 193 bp allele for CsCAT2 locus was the recurrent allele, while the 173 bp, 175 bp, 179 bp, 199 bp, 209 bp, and 215 bp were observed in one or two samples of the 106 samples genotyped. The 106 bp allele of QrZAG75 locus was the most common allele, while the 120 bp allele was the least frequent allele.

Genotyping Cultivars

The combination of SSR profiles across all loci resulted in 61 different chestnut genotypes, when all samples in the study were taken into account (Table 6).

'Colossal': Every sample of cultivar 'Colossal', regardless of the year of planting or the location of the grafted trees in Michigan orchards, consisted of identical SSR profiles across all loci. The SSR profile of every sample of 'Colossal' included in this study resulted in the following SSR profile; 183 bp and 191 bp alleles of CsCAT1 locus, 178 bp and 193 bp alleles of CsCAT2 locus, 124 bp allele of CsCAT16 locus, 76 bp and 88 bp alleles of EMCs15 locus, and 166 bp and 168 bp alleles of QrZAG75 locus (Table 6). To test the reproducibility of the SSR profile of 'Colossal' four leaf samples were collected from a single grafted tree. Each sample represents four cardinal points (north, east, south, west) of the outermost canopy of the grafted tree. The SSR profiles of these four samples were identical to the SSR profile observed for 'Colossal'. The level of uniformity on the SSR profile across all 5 SSR loci among all the 'Colossal' samples included in this study highlights the clonal character of this cultivar and the reproducibility of the methodology.

To determine if grafted 'Colossal' trees could be differentiated from rootstock typically used for its propagation by Fowler Nursery (Newcastle, CA), one leaf sample of one sucker sprout from four different grafted 'Colossal' trees were fingerprinted using all 5 SSR primer sets.

Table 5. Range of the allelic distribution, alleles size, and the frequency per allele for each SSR locus included in this study.

SSR locus name [a]	Alleles $_{obs}$ [b] range (bp)	Frequency [c] alleles (range)	alleles (bp) least – most common
CsCAT1	166 - 222	0.005 - 0.314	166, 181, 189, 195 – 184
CsCAT2	173 -216	0.005 - 0.444	173, 175, 179, 199, 209, 215 – 193
CsCAT16	120 - 171	0.005 - 0.516	120, 129, 131,149 – 124
EMCs15	70-90	0.005 - 0.350	84, 90 – 76
QrZAG75	98 - 168	0.005 - 0.302	102 – 106

a CsCAT= *Castanea sativa* Colture Arboree Torino; EMCs=East Malling UK, *Castanea sativa*; QrZAG=*Quercus robur*, Zentrum fuer Angewandte Genetik, Vienna

b Observed size range (basepairs) of alleles per locus

c Range of frequency of alleles and size of alleles (basepairs) within the frequency range of alleles observed in this study. The list of alleles (bp) is in order from least common to most common.

Upon comparison of the SSR profiles for each sucker sprout, all sucker sprouts could be identified as being genotypically different than 'Colossal'. Two of the 4 rootstock samples could be differentiated from 'Colossal', based on the 175 bp, 195 bp, and 183 bp alleles of CsCAT1, the 194 bp allele of CsCAT2, the 168 bp allele of CsCAT16, the 75 bp and 80 bp alleles of EMCs15, and the 106 bp and 108 bp alleles of QrZAG75. The remaining 2 rootstock samples could be differentiated from 'Colossal' because of the presence of the 70 bp or 76 bp (EMCs15), and the 120 bp and 122 bp (QrZAG75) alleles. Therefore, all 'Colossal could be differentiated from the clonally propagated scion wood grated to them.

'Nevada' and 'Okei': Two ramets of the European x Japanese cultivar 'Nevada', and two grafted trees of the Japanese x Chinquapin cultivar 'Okei' were included in this study because these cultivars were suggested by Fowler Nursery (Newcastle, CA) to efficiently pollinize 'Colossal'. 'Nevada' and 'Okei', were also included in this study because if their SSR profiles could be differentiated from 'Colossal' SSR profiles, one could use these 5 SSR loci as genetic markers in future studies of pollination (*i.e.* crosses) and parentage analysis. The 175 bp allele of CsCAT1, and the 122 bp allele of QrZAG75 were characteristic of 'Nevada'. Furthermore, the 210 bp allele of CsCAT2, and the 127 bp allele of CsCAT16 were typical of 'Okei'. The remaining alleles of EMCs15 in both 'Nevada' and 'Okei' cultivars have been found in other cultivars included in this study, thus these alleles cannot be considered unique.

'Bouche de Betizac', 'Mariguole' and 'Precose Miguole': Grafted trees representing 'Bouche de Betizac', 'Mariguole' and 'Precose Miguole' were added to this study to compare the 'Colossal' SSR profile with these three true French (European x Japanese species) chestnut. Several alleles were present in these cultivars as well as in 'Colossal', for example the 191 bp allele (CsCAT1),

Table 6. Allele sizes (basepairs) at 5 SSR loci of 69 genotypes found in 10 chestnut cultivars grown in Michigan.

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b] CsCAT1 allele size (bp)	CsCAT2 (bp)	CsCAT16 (bp)	EMCs15 (bp)	QrZAG75 (bp)
Benton Harbor	BH ortet	Benton Harbor, MI	170, 184, **182** [c]	189, 193	154, 156	70, 76	104, 106
	BH_ortet_N	Benton Harbor, MI	184, — [d]	189, 193	154, 156	70, 76	104, 106
	BH_ortet_E	Benton Harbor, MI	184, —	189, 193	154, 156	70, 76	104, 106
	BH_ortet_S	Benton Harbor, MI	170, 184, **182**	189, 193	154, 156	70, 76	104, 106
	BH_ortet_W	Benton Harbor, MI	170, 184, **182**	189, 193	154, 156	70, 76	104, 106
	BH_ortet_N (bark)	Benton Harbor, MI	184, —	189, 193	156, —	70, 76	104, 106
	BH_ortet_E (bark)	Benton Harbor, MI	184, —	189, 193	154, 156	70, 76	104, 106
	BH-1	Benton Harbor, MI	176, 184	193, —	124, 156	76, 80	104, 106
	CK-R7T5	Clarksville, MI	184, —	189, 193	124, 156	76, —	104, 106
	BH, MSU-E R5T18	East Lansing, MI	184, —	193, —	124, —	70, 76	104, 106
	BH, MSU-N R5T1	East Lansing, MI	182, 184	193, —	124, 156	70, 76	104, 106
	BH_ortet_S (bark)	Benton Harbor, MI	170, 184, **182**	189, 193	154, 156	70, 76	98, 100
	BH	Benton Harbor,MI	176, 184	189, 193	124, 156	70, 76	104, 106
	BH,"old"MSU-Hort	East Lansing, MI	182, 184	193, —	124, 156	76, —	104, 106
	BH_ortet_W (bark)	Benton Harbor, MI	170, 184, **182**	189, 193	156, —	70, 76	98, 100
	BH graft_SWMREC	Benton Harbor, MI	170, 184, **182**	189, 193	154, 156	70, 76	98, 100
	# 861	Benton Harbor, MI	182, 184	189, 193	156, —	70, 76	98, 106
	EaRpds. poli S BH,	Eaton Rapids, MI	176, 184	193, —	124, 156	70, 76	108, 106
	MSU-E R1T17 BH,	East Lansing, MI	182, 184	193, —	124, 156	80, 81	106, —
	MSU-N R3T8	East Lansing, MI	170, 184, **182**	189, 193	124, —	70, 76	106, —

a Physical location where tree is currently growing and from where sample was collected

b CsCAT= Castanea sativa Colture Arboree Torino; EMCs=East Malling UK, Castanea sativa; QrZAG=Quercus robur, Zentrum fuer Angewandte Genetik, Vienna

c Additional allele typed in bold

d The (—) signs represents either a missing allele that could be of the same size causing an individual to be homozygous at that SSR locus or could be a null allele causing an individual to be heterozygous at that SSR locus.

Table 6. (cont'd)

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b] CsCAT1 allele size (bp)	CsCAT2 (bp)	CsCAT16 (bp)	EMCs15 (bp)	QrZAG75 (bp)
Benton Harbor	CK-R7T6	Clarksville, MI	184, —	189, 193	156, —	70, 76	104, 106
	CK-R7T7	Clarksville, MI	184, —	189, 193	156, 124	70, 76	104, 106
	CK-R7T8	Clarksville, MI	184, —	189, 193	156, —	70, 76	104, 106
	CKR-R2T5	Clarksville, MI	184, —	186, 193	124, —	70, 76	104, 106
	CKR-R2T3	Clarksville, MI	184, —	186, 193	124, —	70, 76	104, 106
Bouche de Betizac	CK-R8T7-'08	Clarksville, MI	184, 191	193, —	128, 127	71, 77	[e]
	CK-R8T14-'06	Clarksville, MI	184, 222	187, —	120, 130	71, 77	104, 106
	CK-R8T16-'06	Clarksville, MI	184, 191	193, —	128, 127	71, 84	104, —
	CK-R4T6	Clarksville, MI	184, 191	179, 193	128, 127	71, 77	104, 106
	# 856	Benton Harbor, MI	184, 222	173, 214	128, 130	77, —	128, —
	BdB_BH	Benton Harbor, MI	184, 191	193, 209	128, 130	77, —	106, —
Colossal	#54	Eaton Rapids, MI	183, 191	178, 193	124, —	76, 88	166, 168
	#55	Eaton Rapids, MI	183, 191	178, 193	124, —	76, 88	166, 168
	#56	Eaton Rapids, MI	183, 191	178, 193	124, —	76, 88	166, 168
	#57	Eaton Rapids, MI	183, 191	178, 193	124, —	76, 88	166, 168
	#58	Eaton Rapids, MI	183, 191	178, 193	124, —	76, 88	166, 168
	R4T1-'92	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168
	R3T15-'97	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168

e The (blank space) indicates missing data.

Table 6. (cont'd)

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b]				
			CsCAT1	CsCAT2	CsCAT16	EMCs15	QrZAG75
			allele size (bp)	(bp)	(bp)	(bp)	(bp)
Colossal	R5T7	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168
	R1 T1	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168
	R1 T2	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168
	R1 T4	Benton Harbor, MI	183, 191	178, 193	124, —	76, 88	166, 168
	1997-1	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1997-2	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1997-3	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1997-4	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1997-5	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1999-1	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1999-2	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1999-3	Owosso,MI	183, 191	178, 193	124, —	76, 88	166, 168
	1999-4	Owosso, MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL1	Clarksville,MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL2	Clarksville,MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL3	Clarksville,MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL4	Clarksville,MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL5	Clarksville,MI	183, 191	178, 193	124, —	76, 88	166, 168
	CL6	Clarksville, MI	183, 191	178, 193	124, —	76, 88	166, 168
	KLF_161_N	Fenton,MI	183, 191	178, 193	124, —	76, 88	166, 168
	KLF_161_E	Fenton,MI	183, 191	178, 193	124, —	76, 88	166, 168
	KLF_161_S	Fenton, MI	183, 191	178, 193	124, —	76, 88	166, 168

Table 6. (cont'd)

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b] CsCAT1 allele size (bp)	CsCAT2 (bp)	CsCAT16 (bp)	EMCs15 (bp)	QrZAG75 (bp)
Colossal	KLF_161_W	Fenton, MI	183, 191	178, 193	124, —	76, 88	166, 168
Colossal (rootstock)	Col.T1A ER	EatonRapids, MI	183, 191	178, 193	124, —	70, 76	120, 122
	Col.MSU-N R2T1	East Lansing, MI	183, 191	178, 193	124, —	76, —	166, 168
	Col.MSU-E	East Lansing, MI	175, 195	193, 194	124, 168	75, 80	106, 108
	R3T15 Col.MSU-E R1T12	East Lansing, MI	175, 183	193, 194	124, 168	75, 80	106, 108
Eaton	Eaton-ortet#1,CT	Connecticut	184, 182	189, —	124, 168	75, 80	106, 108
	Eaton-ortet#2,CT	Connecticut	184, 182	189, —	149, 152	75, 80	106, 108
	Eaton-ortet#3,CT	Connecticut	184, 182	189, —	152, 155	75, 80	106, 108
	Eaton River	Michigan	184, 176			76, —	106, 108
	EaRpds. poli N	Eaton Rapids, MI	184, 182	189, 193	124, 168		106, 108
	Eaton_BH	Benton Harbor, MI	179, —		152, 155		106, 108
	CK-Eaton R9T1	Clarksville, MI	184, 176	189, 180	124, 168	76, —	106, 108
	Eaton 3/29/11	Benton Harbor, MI	184, 176			76, —	106, 108
	Eaton_SWMRE	Benton Harbor, MI	184, —	189, —		76, —	
	C Eaton	Benton Harbor, MI	184, 176	189, 180	124, 168	70, 76	106, 108
	Ea	Benton Harbor, MI	184, 176	189, 180	124, 168	80, 90	106, 108
	CK-R9T2-'06	Clarksville, MI	184, 182	195, 180	124, 168		104, —
	CK-R9T1-'06	Clarksville, MI	184, 182	195, 175	143, 156		104, —

Table 6. (cont'd)

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b]				
			CsCAT1	CsCAT2	CsCAT16	EMCs15	QrZAG75
			allele size (bp)	(bp)	(bp)	(bp)	(bp)
Everfresh	R6T5 ortet_SWMREC	Benton Harbor, MI	182, 184	195, —	124, —	75, 80	106, 108
	CK-R9T13_R6T5 graft	Clarksville, MI	182, 184	195, —	124, —	75, 80	106, 108
	CK-R9T13	Clarksville, MI	182, 184	195, —	124, —	75, 80	106, 108
Everfresh (seedling)	Everfresh_seedling	Benton Harbor, MI	182, 184	195, —	124, —	71, 76	106, 108
	CK-R9T16_seedling	Clarksville, MI	182, 184	195, —	124, —	71, 76	106, 108
J160	R5T5	Benton Harbor, MI	184, 169	193, 189	171, 160	80, 75	106, 98
	# 870	Benton Harbor, MI	184, 169	193, 189	171, 160	80, 75	106, 98
	CK-R6T12	Clarksville, MI	184, 181	193, 178	171, 124	80, 75	106, 98
	J160 3/29/11	Clarksville, MI	184, 183	193, 194	171, 160	80, —	106, 104
	J160	Benton Harbor, MI	184, 183	193, 194	171, 160	75, —	106, 104
	CK-R6T9	Clarksville, MI	183, 189	193, 194	171, 160	71, 77	106, 98
Marigoule	Marigoule	Benton Harbor, MI	185, 191	193, 212	128, 124	77, —	106, 108
	MG TC	Traverse City, MI	185, 191	193, —	128, —	77, 80	106, 108
	MG Don	Traverse City, MI	185, 191	193, —	128, 124	77, —	106, 108

Table 6. (cont'd)

Cultivar/Hybrid	Sample ID	Source [a]	SSR locus [b] CsCAT1 allele size (bp)	CsCAT2 (bp)	CsCAT16 (bp)	EMCs15 (bp)	QrZAG75 (bp)
Nevada	N	Clarksville, MI	175, 191	192, 193	143, 123	82, 85	108, 122
	#860	East Lansing, MI	175, 191	214, 215	143, 141	82, 76	108, 122
Okei	Ok	Eaton Rapids, MI	183, 191	193, 210	127, 123	85, 88	166, 168
	# 863	East Lansing, MI	183, 191	193, 210	127, 129	85, 76	166, 168
Precose Miguole	# 857	Benton Harbor, MI	166, 185	192, 193	143, 142	77, 88	122, 108
	R4T4 7/2/09	Benton Harbor, MI	192, 175	199, 216	143, 142	76, 82	
	# 881	Benton Harbor, MI	192, 185	194, 212	143, 142	77, 88	
	PM "Carmen"_BH	Benton Harbor, MI	185, 191	194, 212		76, —	106, 108
	MSU-R4T19 PM	East Lansing, MI		194, 193			106, 98
	Haack	Traverse City, MI		194, 193	143, —	77, 80	
	PM VR	Leslie, MI		194, 193	143, —	77, 80	
	CK-R9T11	Clarksville, MI		189, 193		75, 80	
	CK-R9T12	Clarksville, MI		189, 195		77, 71	106, 98
	PM TC	Traverse City, MI		193, —	143, —	77, 80	
	Pollock	Traverse City, MI		193, —	143, —	77, 80	
	R5T8	Benton Harbor, MI		216, —	131, —	75, 82	
	CK-P.Migoule R9T12	Clarksville, MI	179, 184		124, 155		

193 bp allele (CsCAT2), and 124 bp allele (CsCAT16). However, the 77 bp allele of EMCs15, and the 106 bp allele of QrZAG75 were found only in 'Bouche de Betizac', 'Mariguole' and 'Precose Miguole'.

'Benton Harbor': Four leaf samples of 'Benton Harbor' ortet and 15 leaf samples of 'Benton Harbor' ramets located at various Michigan orchards were fingerprinted and its SSR profiles compared against the 'Benton Harbor' ortet to determine the genetic variability within the 'Benton Harbor' cultivar. The SSR profile of four leaf samples of 'Benton Harbor' ortet resulted in the following SSR profile; 170 bp, 182 bp, 184 bp alleles of CsCAT1 locus, 189 bp and 193 bp alleles of CsCAT2 locus, 154 bp and 156 bp alleles of CsCAT16 locus, 70 bp and 76 bp alleles of EMCs15 locus, and 104 bp and 106 bp alleles of QrZAG75 locus (Table 6). The 182 bp allele of CsCAT1 locus was detected in the 'Benton Harbor' leaf samples collected from south and west branches of the 'Benton Harbor' ortet, but absent on the leaf samples collected from the north and the east branches. Some alleles were undetected in some bark samples collected from 'Benton Harbor' ortet, for example the 170 bp and 182 bp alleles of CsCAT1 locus in bark samples collected from the north and east branches, and the 154 bp allele of CsCAT16 locus in bark samples collected from north and west branches. The 'Benton Harbor' bark samples consisted of the 98 bp and 100 bp alleles instead of the typical 104 bp and 106 bp alleles of QrZAG75 found in the leaf samples on the same the 'Benton Harbor' ortet.

Several alleles were characteristic of some of the ramets but absent in the 'Benton Harbor' ortet. For example, the 176 bp allele of CsCAT1 was only detected in samples from one orchard in Eaton Rapids and from two unknown orchards (i.e EaRpds.poli S, BH, and BH-1). Leaf samples from seven 'Benton Harbor' ramets located at two MSU research plots in Clarksville and

East Lansing consisted of only the 184 bp allele of CsCAT1 as in the north and east bark samples from the 'Benton Harbor' ortet.

The 189 bp allele of CsCAT2 was undetected in six 'Benton Harbor' ramets, resulting in the only homozygous genotype observed at the CsCAT2 locus. Furthermore, the 186 bp allele of CsCAT2 was only found in two ramets of the 15 leaf samples included in this study.

The 124 bp allele of CsCAT16 was found in 12 'Benton Harbor' ramets, but absent in the SSR profile of the 'Benton Harbor' ortet. Six of the 12 ramets were homozygous at the CsCAT16 locus, from which two ramets shared the same genotype as the north and west bark samples of 'Benton Harbor' ortet.

The 70 bp allele of EMCs15 was undetected in 2 ramets out of the 15 ramets included in this study, resulting in the only two samples with homozygous genotype at this locus. The 80 bp and 81 bp alleles of EMCs15 were found in 2 ramets, which leaf samples were collected at one MSU research plot in East Lansing and from one unknown orchard. The 100 bp allele of QrZAG75 was detected in one 'Benton Harbor' ramet and in the south and west bark samples collected from the 'Benton Harbor' ortet. The 98 bp allele of QrZAG75 was only detected in one ramet, as well as in two of the four bark samples collected from the 'Benton Harbor' ortet. These 100 bp and 98 bp alleles of QrZAG75 were undetected in 2 ramets out of the 15 ramets included in this study, resulting in the only two samples with homozygous genotype at this locus.

'Everfresh': The SSR profile of all leaf samples of 'Everfresh' resulted in the following SSR profile; 182 bp and 184 bp alleles of CsCAT1 locus, 195 bp allele of CsCAT2 locus, 124 bp allele of CsCAT16 locus, and 106 bp and 108 bp alleles of QrZAG75 locus (Table 6). The genetic diversity of 'Everfresh' was only detected with EMCs15 locus, where the 71 bp, 75 bp, 76 bp, and 80 bp alleles were found. The SSR profile of the 'Everfresh' graft used as scion wood for

the two grafted trees included in this study consisted of 75 bp and 80 bp alleles at EMCs15, while two seedling trees of 'Everfresh' included 71 bp and 76 bp alleles.

'J160': Seven grafted trees of 'J160' were included in this study to compare its SSR profile with the SSR profiles of two Chinese selections, 'Benton Harbor' and 'Everfresh'. 'J160' has been considered as a Korean chestnut selection, however there is no true Korean chestnut species but rather hybrids of Japanese species and/or Chinese species. Three alleles were typical of 'Benton Harbor' and 'Everfresh', for example 184 bp allele of CsCAT1, 124 bp allele of CsCAT16, and 106 bp allele of QrZAG75. The 75 bp and 80 bp allele of EMCs15 were found in 5 of the 6 'J160' trees, which were also detected in 'Everfresh', samples of 'Colossal' rootstock and 'Precose Miguole'. Unique 160 bp and 171 bp alleles of CsCAT16 were found in most of the 'J160' grafted trees included in this study.

'Eaton': Samples collected from three chestnut trees considered to be 'Eaton' ortets were identical across 4 of the 5 SSR loci used in this study. Their SSR profiles consisted of 182 bp and 184 bp alleles at CsCAT1, 189 bp allele at CsCAT2, 75 bp and 80 bp alleles at EMCs15, and 106 bp and 108 bp at QrZAG75. Based on CsCAT16 locus, these three 'Eaton' ortets are slightly different due to the presence of 124 bp, 149 bp, 152 bp, 155 bp, and 168 bp alleles (Table 6). The 176 bp (CsCAT1) and 76 bp (EMCs15) were found in the ‘Eaton River’ ramet, which may be unique alleles of EMCs15 for this cultivar.

The designation of grafted 'Eaton' trees planted in Michigan is questionable. Thus, nine leaf samples from grafted trees were included in this study to compare SSR profiles with previously described SSR profiles of 'Eaton' ortets and 'Eaton River' grafted trees (Table 6). The 182 bp and the 184 bp alleles at CsCAT1 were found in two grafted trees planted in Clarksville, and one grafted tree planted in Eaton Rapids. However, the 176 bp allele (CsCAT1) and the 76

bp (EMCs15) were found in three grafted trees planted in the MSU research plots in Clarksville, instead of the 182 bp allele (CsCAT1), and the 75 bp and 80 bp alleles (EMCs15) present in the 'Eaton' ortets. Two grafted trees were found to be homozygous at the CsCAT1 locus, where one tree consisted of the 184 bp allele and the second tree the 179 bp allele. The 106 bp and 108 bp alleles at QrZAG75 were found on 7 of the 9 grafted trees included in this study. The remaining 2 of the 9 grafted trees were homozygous at the QrZAG75 locus, where only a 104 bp allele was detectable.

Characteristics of SSR profiles Based on Putative Species

After grouping all samples into general categories based on putative pedigrees (*i.e.* Chinese hybrid, European x Japanese hybrid, Chinese x Japanese x American hybrid, and Japanese x Chinquapin hybrid), the SSR profiles of the majority of the cultivars within each putative pedigree had various alleles in common. The SSR profile of most of the cultivars considered as Chinese hybrids consisted of the 184 bp (CsCAT1), 189 bp (CsCAT2), and 75 bp or 80 bp (EMCs15) alleles. Two exceptions were found to this generality. When Chinese hybrid SSR profiles were observed; the 189 bp allele of CsCAT2 was absent in samples of 'Everfresh', and neither the 75 bp and 80 bp were present in samples of 'Benton Harbor'. The SSR profile of most of the cultivars identified as European x Japanese hybrids included the 191 bp (CsCAT1), 193 bp (CsCAT2), 124 bp (CsCAT16), and 76 bp or 77 bp (EMCs15) alleles. Two exceptions to this general European x Japanese hybrids SSR profile were observed; (1) the 191 bp allele of CsCAT1 was not found in samples of 'Precose Miguole', (2) the 124 bp allele of CsCAT16 was not detected in samples of 'Bouche de Betizac', and 'Nevada'. The 106 bp and 108 bp alleles of QrZAG75 were found in the majority of the samples thought to be European x Japanese hybrids and Chinese hybrids, but these two alleles were absent in all samples of the European x Japanese

hybrid 'Colossal'. Because the Japanese x Chinquapin hybrid and the Chinese x Japanese x American hybrid were represented in this study by a single cultivar each, the description of the SSR profiles of the cultivars in these two groups should be considered preliminary. The Japanese x Chinquapin cultivar, 'Okei' had a unique SSR profile where the 210 bp (CsCAT2), and the 123 bp, 127 bp, and 129 bp (CsCAT16) alleles considered as the 'Okei' genotype. However, the majority of the remaining alleles found in 'Okei' SSR profile were also part of the European x Japanese hybrids, mainly in 'Colossal'. The majority of the samples considered as 'Eaton' cultivars consisted of unique 149 bp, 152 bp, 155 bp, and 168 bp alleles for CsCAT16 locus. The remaining alleles of 'Eaton' cultivars were also found in the majority of the samples considered as Chinese hybrids.

In summary, a limited number of rare or unique SSR alleles were identified for 5 of the 11 cultivars included in this study (Table 7). The 170 bp allele of CsCAT1 and the 154bp allele of the CsCAT16 allele were found in the majority of the samples of ‘Benton Harbor’, a Chinese selection. However, CsCAT1 and CsCAT16 loci did not result in unique alleles in ‘Everfresh’ samples, the other Chinese selection included in this sample, as it did in ‘Benton Harbor’ samples. A 195 bp allele of CsCAT2 was observed to be unique in all the ‘Everfresh’ samples included in this study (Table 7). The European x Japanese cultivar, ‘Colossal’ had a unique SSR profile where the 178 bp allele of CsCAT2 and the 88 bp allele of EMCs15 were observed in every sample. However, the ‘Nevada’ samples, also a European x Japanese cultivar, had a 175 bp allele of CsCAT1, which made ‘Nevada’ SSR profile unique. As mentioned above, ‘Okei’ SSR profile obtained with CsCAT16 locus shared three alleles with the SSR profile with the European x Japanese hybrid samples in this study, however the 210 bp of CsCAT2 is truly

Table 7. Summary of unique alleles in the SSR profiles in 5 chestnut cultivars of the 11 chestnut cultivars included in this study.

Hybrid	**Cultivar**	**SSR loci [a]**	**Allele (bp) [b]**
Chinese	'Benton Harbor'	CsCAT1	170
		CsCAT16	154
	'Everfresh'	CsCAT2	195
European X Japanese	'Colossal'	CsCAT2	178
		EMCs15	88
	'Nevada'	CsCAT1	175
		QrZAG75	122
Japanese X Chinquapin	'Okei'	CsCAT2	210
		CsCAT16	127

a CsCAT= *Castanea sativa* Colture Arboree Torino; EMCs= East Malling,UK, *Castanea sativa*

b Allele observed in basepairs (bp)

unique to the ‘Okei’ cultivar. Overall, the SSR locus CsCAT2 resulted in the higher number of unique allele (3 alleles in 3 cultivars out of 5 cultivars with unique SSR alleles) in this data set, while the SSR loci EMCs15 and QrZAG75 produced the lower number of unique alleles (1 allele in 1 cultivar out of 5 cultivars with unique SSR alleles).

Genetic Relationships Among Cultivars/Hybrids

The phenogram constructed for the 110 chestnut samples representing putative pedigrees of four cultivars (i.e. Chinese hybrids, European x Japanese hybrids, Chinese x Japanese x American hybrid, and Japanese x Chinquapin hybrid) grouped the cultivars into two major clusters (*i.e*. A and B) and two minor clusters within the major cluster A (*i.e*. A-1 and A-2) (Figure 3). When all individuals were genotyped as homozygotes consisting of two copies of the same allele, cultivars considered as Chinese hybrids, 'Benton Harbor' and 'Everfresh' and 'J160' belong to the major cluster A, yet 'Benton Harbor' and 'Everfresh' grouped forming the minor cluster A-1 and 'J160' in cluster A-2 (Figure 3). 'Eaton' considered as Chinese x Japanese x American hybrid also grouped with 'Benton Harbor' and 'Everfresh' in cluster A-1. Cultivars considered as European x Japanese hybrids, 'Bouche de Betizac', 'Mariguole', and 'Precose Miguole' grouped forming the minor cluster A-2, together with the questionable Korean hybrid 'J160'. Cultivars considered as European x Japanese hybrids and propagated by the same Californian nursery (Fowler Nurseries), 'Colossal' and 'Nevada' were found forming the second major cluster, cluster B. The Japanese x Chinquapin hybrid, 'Okei' also propagated by Fowler Nursery grouped with 'Colossal' and 'Nevada'. However, 'Colossal' grouped slightly different than 'Nevada' and 'Okei', as it formed its own group with samples of 'Colossal' rootstock.

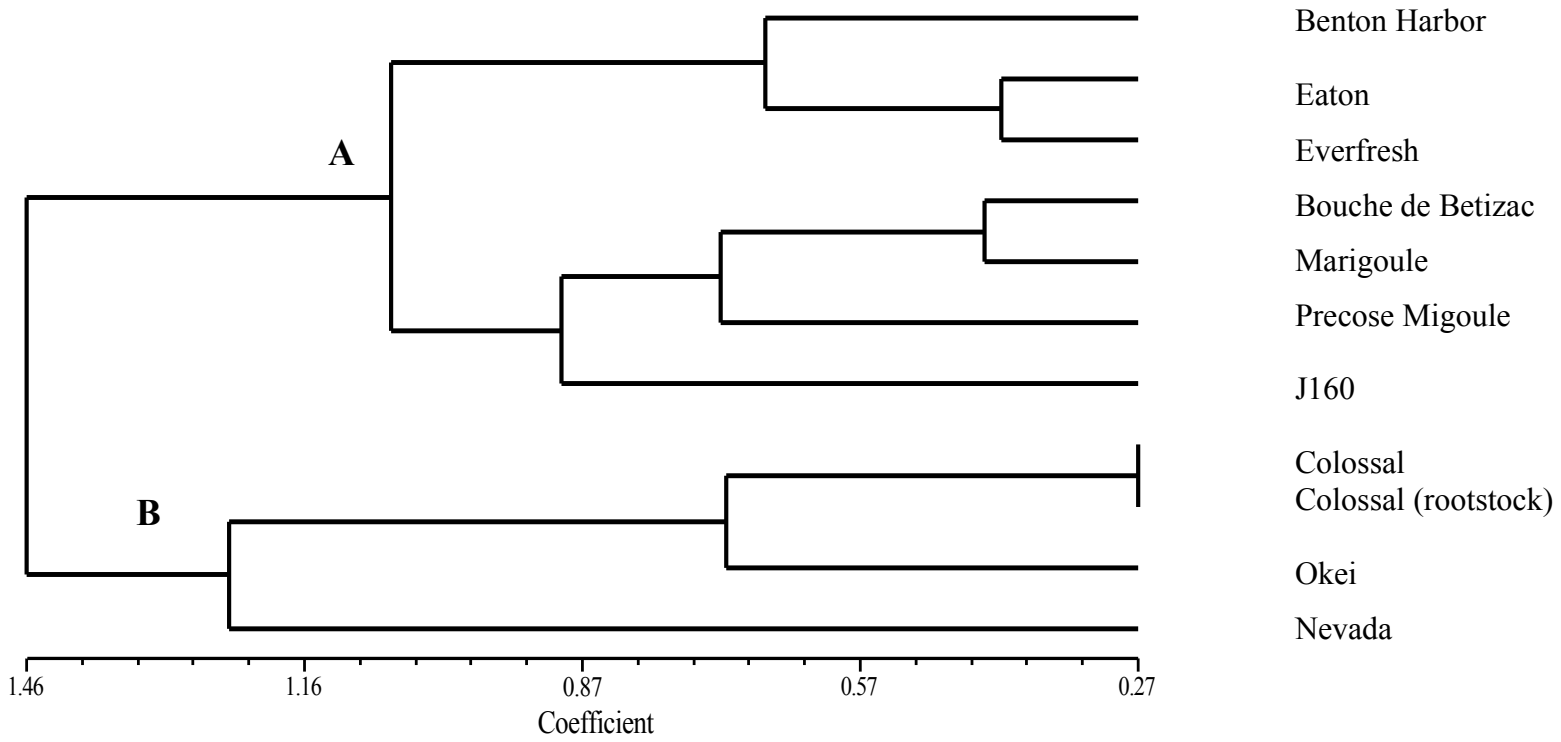


Figure 3. Phenogram of the 110 chestnut genotypes evaluated in this study. The phenogram was constructed using UPGMA method based on genetic distance (Nei, 1972) calculated with POPGENE software. Data set includes genotype for homozygotic individuals (2N) as consisting of two alleles of the same allele size. Letters (A and B) represent major and minor clusters, respectively.

SSR-based Evaluation of Progeny from an Open-pollinated Orchard in Michigan

To test the efficacy of SSR primers as genetic markers paternity analysis was conducted for the paternal trees and the F1 pregeny (*i.e.* nuts) of a tri-parental cross among 'Colossal' (maternal parent), and ‘Benton Harbor’ and ‘Okei’ (paternal parents). DNA was extracted for each nut after kernel tissue from each nut was sampled following a bone marrow biopsy assay.

The SSR-based identification of individuals could only be performed if the SSR alleles of the parental trees involved in the cross were known and if at least one allele of the SSR profile of the parental cultivar was partially unique to each cultivar. The SSR profiles consisting of alleles at the EMCs15 and CsCAT1 loci of the individuals involved in the tri-parental cross and its F1 progeny (*i.e.* nuts) were sufficient to identify the paternal parent (*i.e.* pollinizer tree) of each nut harvested from this cross.

Pollination

A total of 53 burs were recovered from the 120 ‘Colossal’ female flowers naturally pollinized by a single ‘Benton Harbor’ tree or by a single ‘Okei’ tree. In 2010, pollination of receptive ‘Colossal’ female flowers occurred as early as 23-June at the Eaton Rapids orchard. Out of the 53 burs, fourteen (14) nuts were recovered from ‘Colossal’ flowers exposed to airborne pollen by 10-July, and 42 nuts were recovered from ‘Colossal’ flowers exposed on 12-July (Table 8). Fourteen nuts were recovered from the 60 ‘Colossal’ female flowers included in the study as control, which were exposed to airborne pollen through the entire blooming period of the maternal trees (‘Colossal’) and the paternal trees (‘Benton Harbor’ and ‘Okei’). In support of anecdotal observations on chestnut phenology, the majority of the ‘Okei’ pollen was mature and airborne at the same time as all three-style groups on the majority of the ‘Colossal’ female flowers were fully elongated. On 23-June, the majority of the catkins on the 8-years old ‘Okei'

Table 8. Effect of pollination bag on the total number of nuts harvested from ‘Colossal’ trees. Nuts harvested were the result of a tri-parental cross, where European x Japanese chestnut cultivar ‘Colossal’ served as the maternal tree, and the Chinese cultivar ‘Benton Harbor’ and the Japanese x Chinquapin hybrid ‘Okei’ served as pollinizer trees.

Treatment—Bag placed on flower [a]	**Bag removed from flower** [b]	**Total number of nuts** [c]
(date)	(date)	
20-Jun	**23-Jun**	14/70
10-Jul	**late August**	
20-Jun	**12-Jul**	42/70
n/c [d]	**12-Jul**	
__ [e]	**—**	14/70

a One hundred twenty female flowers in a total of four 'Colossal' were covered with pollination bags (Lawson #451) on 20-June during 2010 season. Pollination bag placed on ‘Colossal’ female flowers prevents pollination from the date of its placement over the flowers.

b Sixty (60) of the one hundred twenty ‘Colossal’ female flowers previously covered with pollination bags (Lawson #451) on the indicated dates were removed. Pollination bags removed from ‘Colossal’ female flowers allows pollination to takes place from the date the bag was removed through the remaining blooming period.

c Total number of nuts collected per date out of the total 70 nuts collected for the entire study.

d n/c = not conducted. These 60 'Colossal' female flowers were not bagged a second time after the 12-July and remained exposed to airborne pollen during the entire blooming time and pollen dehiscence of either 'Benton Harbor' or 'Okei' trees.

e — represents 60 'Colossal' female flowers included in the study, which serve as control. These flowers were not bagged at any time during blooming period or pollen dehiscence of either 'Benton Harbor' or 'Okei' trees.

tree at this Eaton Rapid orchard were longistaminate with fully functional pollen. Thus, the 'Okei' pollen dehiscence duration and the 'Colossal' female flowers receptivity period seem to have a more synchronized timing for flowers to be pollinized, than the timing between the peak time of 'Benton Harbor' pollen dehiscence and the 'Colossal' female flowers. Due to the abundant number of flowers on 'Colossal' trees and the longevity of receptive period of 'Colossal' flowers (*i.e*. late-July), airborne pollen from the 'Benton Harbor' tree was capable to pollinized as many flowers as the pollen from the 'Okei' tree (Table 8). Furthermore, although the majority of the catkins on the 4-years old 'Benton Harbor' tree at this Eaton Rapid orchard were immature on 23-June, the precocity and robustness of this cultivar and its pollen were sufficient to pollinate 'Colossal' female flowers. It seems that the partial number of fully matured mesostaminate catkins on the 'Benton Harbor' tree did not hinder the potential of this cultivar ('Benton Harbor') as an efficient pollinizer of 'Colossal' female flowers.

Simple Sequence Repeats (SSR) analysis of Parental Cultivars and F1 progeny

Of the total 120 'Colossal' flowers included in the study, 70 nuts were successfully pollinized by either 'Benton Harbor' or 'Okei' pollen. Of these 70 nuts, DNA was successfully extracted from 69 seeds (*i.e*. kernels) using the kernel biopsy method developed for this study. The SSR genotype of the parental trees and the progeny was conducted in two stages; all nuts were genotyped using EMCs15, and nuts with ambiguous genotype were subjected to a second run of SSR-based evaluation using CsCAT1. All three parental cultivars were heterozygous at the EMCs15 locus (Table 3). All four 'Colossal' trees consisted of a 76 basepair (bp) and a 88 bp alleles at EMCs15 locus. One should expect, that upon individual assortment of alleles and segregation, each nut (*i.e.* F1 progeny, individual) should harbor either the 76 bp or the 88 bp

EMCs15 allele inherited from the maternal parent, 'Colossal'. Since chestnuts are diploid, each gene must carry two alleles, thus the question still remains, where did the other EMCs15 allele of each nut originated from, 'Benton Harbor' or 'Okei'? The genotype of the 'Benton Harbor' tree consisted of a 70 bp and a 76 bp alleles at the EMCs15 locus, while the genotype of the 'Okei' tree consisted of an 85 bp and an 88 bp (Table 3). As an exercise, one might expect that a nut with a homozygous genotype at the EMCs15 locus, consisting of only a single 76 bp allele (s) at the EMCs15 locus indicate that this nut was pollinized by 'Benton Harbor'. Similarly, another nut with a homozygous genotype at the EMCs15 locus, consisting of only a single 88 bp at the EMCs15 locus, indicates that this nut was pollinized by 'Okei' rather than by 'Benton Harbor'. Since the 70 bp allele at the EMCs15 locus is a "unique allele" for 'Benton Harbor' and it can serve as a genetic marker for inheritance derived from 'Benton Harbor', every nut with a heterozygous genotype at the EMCs15 locus containing the 70 bp allele as one of the pair of alleles (*i.e.* 70,76 and 70,88 genotypes) must have been pollinized by 'Benton Harbor'.

Similarly, the 85 bp allele at the EMCs15 locus is a "unique allele" for the 'Okei' cultivar, and it can also serve as a genetic marker for the F1 progeny obtained from this cross. Every nut with an heterozygous genotype at the EMCs15 locus containing the 85 bp allele as one of the pair of alleles (*i.e.* 76,85 and 85,88) must have been pollinized by 'Okei'. Ambiguity rises when one analyzes nuts harboring the 76 bp and 88 bp alleles at the EMCs15 locus. A nut with the following heterozygous genotype at the EMCs15 locus (76,88) and recovered from this specific trio of parental cultivars ('Colossal'; maternal parent, and 'Benton Harbor or 'Okei' paternal parents), could not be identified solely by its EMCs15 allelic pattern because this combination of alleles could result from either paternal parent (*i.e*. pollen donor), 'Benton Harbor' or 'Okei' (Table 3).

All expected genotypes at the EMCs15 locus were observed in the F1 progeny (*i.e*. nuts) from the cross among the three parental cultivars ('Colossal', 'Benton Harbor' and 'Okei') included in this study (Table 3 and Table 9). The distribution of the nuts per genotype observed was as follow. Twenty-five (25) out of 70 nuts recovered were homozygous at the EMCs15 locus; where 15 of the 25 nuts locus contained the 76 bp allele and the remaining 10 nuts (10 out of 25) contained the 88 bp allele (Table 9). Nuts containing the 76,— genotype resulted from 'Colossal' flowers pollinized by 'Benton Harbor' pollen, while nuts containing the 88,— genotype resulted from 'Colossal' flowers pollinized by 'Okei' pollen.

A total of 45 nuts resulted to be heterozygous at the EMCs15 locus (Table 9). The heterozygous genotype most frequently observed in the F1 progeny (*i.e*. nuts) was (76,85), immediately followed by (76,88). The majority of the nuts recovered, 16 out of 70 nuts, as well as the majority of nuts with heterozygote genotype (16 out of 45 nuts), had the 76 bp and 85 bp alleles at the EMCs15 locus. Nuts with the 76,85 genotype resulted from 'Colossal' flowers pollinized by 'Okei' pollen. The third largest number of nuts recovered with heterozygote genotype (11 out of 45 nuts), had the 85 bp and 88 bp alleles at the EMCs15 locus. Nuts with the (85,88) genotype resulted from 'Colossal' flowers pollinized by 'Okei' pollen. The first to lowest number of nuts (3 out of 45 nuts) with a heterozygote genotype contained the 70 bp and the 76 bp alleles, and the lowest number of nuts (1 out of 45 nuts) contained the 70 bp and the 88 bp alleles at the EMCs15 locus. Nuts with the 70,76 or the 70,88 genotype resulted from 'Colossal' flowers pollinized by 'Benton Harbor' pollen.

Of these 45 nuts, 14 nuts contained the 76 bp and 88 bp alleles at the EMCs15 locus, unfortunately, the presence of these alleles at this locus was not enough to determine which cultivar pollinized the 'Colossal' flowers from which these nuts developed (Table 9). To solve

Table 9. Distribution of the nuts (*i.e.* F1 progeny) per SSR genotype.

SSR genotype [a]		Total number of nuts [b]	Nut (kernel) identification	Pollinizer based on SSR genotype [c]	
EMCs15	**CsCAT1**			**'Benton Harbor'**	**'Okei'**
70,76		3/70	4, 67, 71	yes	no
70,88		1/70	13	yes	no
76,— [d]		15/70	22, 6, 1, 65, 27, 34, 37, 12, 50, 52, 66, 61, 9, 68, 30	yes	no
76,88 [e]		**14/70**	57, 70, 39, 29, 31, 8, 32, 38, 43, 64, 54, 69, 18, 19	not determined until genotyped with CsCAT1	

a *Castanea* species are diploid, thus each SSR genotype be carrying two alleles at each SSR locus. SSR alleles are described based on base pair length. EMCs = East Malling UK, *Castanea sativa* (Buck et al., 2003); CsCAT = 2003) Colture *Castanea sativa* Arboree Torino (Marinoni et al., 2003).

b Number of nuts recovered from 180 'Colossal' female flowers naturally pollinized by 'Benton Harbor' or 'Okei' in a study performed at an orchard in Eaton Rapids, Michigan (Eaton County) in 2010.

c yes, represents the participation of the cultivar as a pollen donor based on visual analysis of the SSR genotype of the nut (i.e. F1 progeny), while no represents the exclusion of the cultivar as pollen donor.

d — represents a homozygous genotype, where an individual carries two alleles of the same size (basepairs).

e The inheritance of the 76 bp and the 88 bp alleles, conferring an heterozygous genotype at the EMCs15 locus, to a nut cannot be determined solely on this SSR marker. Nuts with this genotype were further analysed using CsCAT1 primer set.

Table 9. (cont'd)

SSR genotype [a]		Total number of nuts [b]	Nut (kernel) identification	Pollinizer based on SSR genotype [c]	
EMCs15	**CsCAT1**			**'Benton Harbor'**	**'Okei'**
76,85		16/70	21, 3, 28, 42, 35, 59, 5, 33, 36, 44, 24, 25, 17, 63, 47, 48	no	yes
85,88		11/70	2, 62, 60, 10, 45, 53, 23, 49, 40, 46, 45		
88,—		10/70	58, 7, 20, 11, 15, 14, 51, 16, 41, 56		
76,88		**14/70**	same as above	not determined until genotyped with CsCAT1	
	183,184	1/15	57	yes	no
	184,191	2/15	31, 38	yes	no
	176,183	0/15			
	176,191	0/15			
	183,191	8/15	64, 39, 70, 43, 69, 18, 19, 8	no	yes

Table 9. (cont'd)

SSR genotype [a]		Total number of nuts [b]	Nut (kernel) identification	Pollinizer based on SSR genotype [c]	
EMCs15	CsCAT1			'Benton Harbor'	'Okei'
	183,—	1/15	54	no	yes
	191,—	2/15	29, 32	no	yes

the ambiguity observed on these 14 nuts with the 76 bp and 88 bp alleles at the EMCs15 locus, the 14 nuts were genotyped using CsCAT1 primer pair. The genotype observed for the paternal trees was as follow. All four 'Colossal' trees were heterozygous at the CsCAT1 locus and contained 183 bp and 191 alleles (Table 9). Likewise, the genotype of the 'Okei' tree was heterozygous at the CsCAT1 locus and contained the 183 bp and the 191 alleles, same as the 'Colossal' cultivar. In a scenario where any other SSR primer set where to be used to genotype the F1 progeny resulting from this parental cross (*i.e.* 'Colossal' crossed with either 'Benton Harbor' or 'Okei'), CsCAT1 could have not been useful for paternity assignment. However, all of these 14 nuts were previously genotyped with the EMCs15 primer set, and the SSR profile of these nuts were a combination of the results from these two SSR primer sets. By visually analyzing the combined SSR profile of eat nut, the overall informative value and usefulness of these SSR primer sets to be used for paternity analysis increased. The 'Benton Harbor' tree was also heterozygous at the CsCAT1 locus and consisted of 176 bp and 184 bp alleles (Table 8).

Four (4) out of the possible 7 genotypes at the CsCAT1 locus were observed (Table 9). The two genotypes not found on this F1 progeny (*i.e.* nuts) were: the heterozygous genotypes carrying the alleles 176,183 and 176,191. The distribution of the nuts per genotype observed was as follow. Eleven out (11) of the 15 nuts were heterozygous while 3 nuts were homozygous at the CsCAT1 locus, and the genotype of the remaining nut of the 15 nuts was not determined. Of these 11 nuts, 8 nuts heterozygotes at the CsCAT1 locus contained the 183 bp and the 191 bp alleles, 2 nuts contained the 184 bp and the 191 bp alleles, and the remaining nut (1 out of 15) contained the 183 bp and the 184 bp alleles. Nuts with the 183,191 genotype resulted from 'Colossal' flowers pollinized by 'Okei' pollen while nuts with the 184,191 or the 183,184 genotypes were pollinized by 'Benton Harbor' pollen.

A total of 3 nuts (3 out of 15) were homozygous at the CsCAT1 locus (Table 9). Two out of the 3 homozygous nuts contained the 191 bp allele, and only 1 nut of the 3 homozygous nuts contained the 183 bp allele. Nuts with the 191,— or the 183,— genotype resulted from 'Colossal' flowers pollinized by 'Okei' pollen. Due to the low number of nuts (*i.e*. F1 progeny) collected in this study, fitness of the observed genotypes at each SSR locus (EMCs15 and CsCAT1) to the expected genotypes at each SSR locus could not be tested using Chi-square.

Microsatellite Analysis

The values of observed (Ho) and expected heterozygosity (He) for EMCs15 locus were 0.657 and 0.656, respectively. Identical values for Ho and He suggests that the chestnut population in this data set is at Hardy-Weinberg equilibrium, however, these values indicate that the frequency of all the alleles in the data set is equal. Because the frequency of alleles is this data set is not equal, the identical values for Ho and He suggest that this population is not experiencing the effect inbreeding and that the population did not originated from two independent populations that are under the current conditions allowed to mix (isolate-breaking effect). The average value of the polymorphic information content (PIC) was 0.546, which suggests that the markers involved in determining the genotype of the F1 progeny were highly informative. The average paternity exclusion probability (PE) observed was 0.416, and the average probability of identity (PI) observed was 0.009. PE refers to the probability of excluding a random individual as a potential parent of the individual based on the genotype of one parent and the offspring. PI is the probability of two unrelated individuals showing the same multilocus genotype by chance. Both PE and PI values obtained from this data set might suggest that SSR markers selected for this study are slightly useful for parentage analysis, however, these values cannot be used to determine the efficacy of these SSR markers because this data consisted of

only two SSR markers per individual (*i.e*. F1 progeny and parental trees), which is not considered a robust SSR profile for an individual, and a small sample size. However, because this study was conducted in a controlled fashion, the significance of the PE and PI values is superfluous.

DISCUSSION

Michigan has a great diversity of chestnut germplasm partially due to the introduction of Chinese (*C. mollissima*) and other species during the 20th century, the excessive use of seedling chestnuts in orchards, and the accidental hybridization events that may have occurred among introduced species and wild, naturalized populations of American chestnut (*C. dentata*) planted during the late 1800s (Fulbright et al, 2010). The recent introduction of cultivars of European x Japanese hybrids and Chinese cultivars, has, in some cases exacerbated the diversity because some growers now plant the resulting seedlings from these trees. That is, some growers see nothing wrong with planting nuts from a European x Japanese hybrid that was pollinized by a Chinese chestnut seedling tree giving rise to nuts with a background of (*C. sativa* x *C. crenata*) x *C. mollissima*. It can become even more complex when a row of 'Colossal' (European x Japanese hybrid) is pollinized by a row of 'Eaton' trees, Japanese x American (*C. crenata* x *C. dentata*) x Chinese (*C. mollissima)*, meaning the harvested nuts could be the result of (*C. sativa* x *C. crenata*) x (*C. crenata* x *C. dentata*) x *C. mollissima*. Unfortunately, some growers will not only grow these nuts in their orchards, they may distribute the resulting seedlings. I have observed very stunted poor growing seedlings in the greenhouse from some of these complex crosses. Although I did not identify any of the SSR loci used in this study as a genetic marker for the stunted tree phenotype, it would be intriguing to discover the genotypic change that cause such apparently small "runted" trees.

A newly discovered affliction leading to a kernel breakdown in marketable chestnut has been observed on more than 30 percent of the 'Colossal' nuts when pollinized by Chinese chestnut cultivars and seedlings and by the cultivar 'Okei'. Studies eventually leading to answers to these intriguing questions will be approachable as we learn more about the genetics of the various species and cultivars and their resulting offspring.

This chapter is mainly dedicated to finding genetic markers that might be useful in following the genomes of various species and cultivars. The same techniques will also be useful in "policing" nurseries and orchards to guarantee that nurseries are selling what they say they are selling and growers are not trying to produce nuts from rootstock that long ago lost its grafted scion and then re-sprouted from rootstock. To accomplish the long-term goal of an accurate description of chestnut germplasm, it was necessary to establish a reliable method for the identification and validation of species, hybrids and cultivars currently growing in Michigan orchards. Thus, the main objective of this study was the identification of 11 chestnut cultivars using simple sequence repeat (SSR) markers.

SSR-based genotyping has been extensively applied for the identification and validation of European, Japanese, and Chinese cultivars and the description of the level of genetic diversity in European, Japanese, and Chinese germplasms (Martin et al, 2010a; Martin et al, 2010b; Martin et al, 2010c; Nishio et al, 2011; Wang et al, 2008; Yamamoto et al, 2003).

SSR marker analysis has been accepted as one of the main DNA-based fingerprinting methodology of characterizing hybrids among *Castanea* species, due to the high level of polymorphism that co-dominant SSR markers can reveal. Various species-specific SSR markers have been developed for *Castanea* species (Kampfer et al, 1998; Buck et al, 2003; Marinoni et al, 2003; Nishio et al, 2011), and some of these have been useful for identification of individuals

across genera within the *Fagaceae* family (Akkak et al, 2010; Beccaro et al, 2005; Boccacci et al, 2004; Botta et al, 2005; Costa et al, 2005; Cuenca et al, 2010). Furthermore, successful cross-species amplification using SSR markers had been essential for genetic mapping (Barreneche et al, 1998; Casasoli et al, 2001), comparative mapping (Barreneche et al, 2003), and phylogeny of species (Aldrich et al, 2003).

Usefulness of SSR as Genetic Markers

As expected, all 4 SSR primer pairs developed for *C. sativa* (Buck et al, 2003; Marinoni et al, 2003) resulted in positive amplification of all European x Japanese hybrids (*C. sativa* x *C. crenata*) tested. This could be interpreted as follows, the flanking regions of each of these 4 SSR loci remained unchanged in the European-Japanese hybrids regardless of the chestnut species for which these primers were originally designed to identify. However, to determine if the resulting SSR fragments are allelic, one would need to compare the sequences of the fragments and the flanking regions of the samples tested, and the *C. sativa* cultivars used to design each primer set. An alternative way to test for the allelic character of the SSR fragments is to perform controlled crosses and determine if the F1 progeny (*i.e*. offspring/seeds/nuts) inherited the alleles in question (*personal data*, included in this chapter, below). The presence of SSR loci across the variety of species and hybrids of *Castanea* is not a surprise, as previous comparative genetic mapping studies have revealed the location of CsCAT1, CsCAT2, and EMCs15 on three of the 12 linkage groups of American, Chinese, and European chestnut (Sisco et al, 2005). This study demonstrated the usefulness of these SSR markers as genotypic characters, providing unique SSR profiles in the identification of *Castanea* hybrids.

One SSR primer pair developed from oak (*i.e*. QrZAG75) resulted in positive amplification for *Castanea* hybrids tested in this study. These results support previous findings

(Akkak et al, 2010; Boccacci et al, 2004) regarding the homology between *Quercus* and *Castanea* genomes. However, alleles of EMCs15 and QrZAG75 resulted in repeats of smaller lengths than previously reported. It is possible that lower number of basepairs of the alleles found in the majority of the *Castanea* hybrids tested could potentially be an outcome of template slippage and\or mutations in the flanking regions of the SSR locus, which are causes for the variability observed in microsatellite-based genotyping (Powell et al, 1996).

Recently, Nishio et al (2011) revealed a "new" set of SSR markers capable of identifying common *C. mollissima* cultivars and European x Japanese hybrids grown in Japan. Japanese germplasm appeared to be quite diverse, consisting of intensively cultivated *C. crenata,* and *C. mollissima* cultivars as well as recently introduced Japanese x Chinese hybrids. It would be beneficial to evaluate this "new" set of SSR markers in the cultivars/hybrids currently grown in Michigan because these SSR primers were designed and tested on Chinese (*C. mollissima*) and Japanese (*C. crenata*) species, unlike the SSR markers already tested in this study which where designed for European (*C. sativa*) species. In addition, Michigan's germplasm is predominantly composed of European x Japanese hybrids and Chinese hybrids. Furthermore, experts have demonstrated the efficiency of combining loci for the identification of chestnut cultivars, where a high level of resolution is needed to generate unique SSR profiles (Cantini and Autino 2010; Costa et al, 2004; Cuenca et al, 2010; Martin et al, 2004; Martin et al, 2010b; Mattioni et al, 2009).

SSR loci Polymorphism

Unique SSR profiles were observed for 5 of the 11 cultivars included in this study: ‘Benton Harbor’, ‘Everfresh’, ‘Colossal’, ‘Nevada’, and ‘Okei’. In this study, an unique SSR profile was defined as the presence of rare or unique alleles in 1 or more SSR loci. Although the

identification of rare SSR alleles in a cultivar may result in an advantageous tool for the use of the SSR marker in future genotyping experiments, in diploid individuals like chestnuts, the identification of rare alleles in one SSR locus is not sufficient for the identification of unknown parentage. In theory, in a diploid individual, an SSR profile based on a minimum of three SSR loci should be able to distinguish 11 cultivars. The genotype of a diploid individual could be one of three possibilities, homozygous for each of the two co-dominant alleles or heterozygous (i.e. aa, ab, and bb) for each SSR loci. Thus, three SSR loci will have a maximum of 27 (3 to the power of 3) possible combinations of the genotypes, which is greater than 11. To use less than 3 SSR loci, that is, one SSR locus or 2 SSR loci will result in a maximum of 3 or 9 possible combinations of the genotypes, respectively, which would be a lower number than the number of cultivars intended to be identified in this study (*personal communication*, Dr. Dechun Wang; MSU-Department of Crop and Soil Sciences). Hypothetically, an unknown chestnut sample could be identified as 'Colossal' if its SSR profile was based on the presence of 183bp of CsCAT1, 178 bp of CsCAT2, 88 bp of EMCs15, and 166 or 168bp of QrZAG75 alleles. The same unknown chestnut samples could be identified as 'Benton Harbor' if its SSR profile contains all the following alleles; 170 bp (CsCAT1), 189 bp (CsCAT2), 154 bp or 156 bp (CsCAT16), 70 bp (EMCs15), and 98 bp, or 100 bp, or 104 bp (QrZAG75). The unknown chestnut sample could be considered as 'Eaton' if in its SSR profile the 180 bp (CsCAT2), and 149 bp, or 152 bp, or 155 bp, or 168 bp (QrZAG75) alleles were found. Instead, the same unknown chestnut sample could be identified as 'Nevada' if its SSR profile consisted of the 175 bp (CsCAT1) and 122 bp (QrZAG75), or as 'Okei' if the 210 bp (CsCAT2) and 127 bp (CsCAT16) alleles were found. However, this data set is limited in the number of unique alleles that could be used to identify an unknown chestnut sample as 'Bouche de Betizac', 'Mariguole',

'Precose Miguole', 'Everfresh', and 'J160' because only one SSR locus resulted in unique alleles among these cultivars.

Regardless of the high level of polymorphism that could be achieved with these 5 SSR loci, it is noteworthy the low number of effective alleles (3.4 to 5.4) obtained per each SSR locus. As an example, low number of effective alleles (3.4) suggests that 3 to 4 alleles of the total number of alleles of CsCAT16 observed (*i.e*. 19) are needed to obtain the same level of heterozygosity observed with CsCAT16 locus. In general, a low number of effective alleles is an indication of the high level of homozygotic individuals genotyped with one genetic marker and observed in the samples tested.

Due to the high level of homonymy observed in 10 of the 11 cultivars, excluding 'Colossal' cultivar and the 'Colossal' rootstock (cultivar unknown), the clonal character by which grafted trees are recognized as cultivars becomes questionable. Research containing data regarding the mutation rate of SSR in chestnuts is not currently available, however, high levels of SSR mutation rates (10^{-4}, 10^{-3}, and 10^{-2}) have been determined in wheat (Thuillet et al, 2002) and chickpea (Udupa and Baum 2001). It is possible that the mutational rate of simple sequence repeats in chestnuts is greater than anticipated. High levels of homonymy within clones have been previously reported in hybrid poplar cultivars (Rathmacher et al, 2009). Similarly as what could have occurred with the description of chestnut cultivars, many of the cultivated poplar trees are selected from a set of seedlings that were formed upon the natural hybridization process between two *Populus* species, *P. nigra* and *P. deltoides.* In poplar, *P. nigra* and *P. deltoides* consist of multiple subspecies, which depending on the genotype of these subspecies, one could potentially name a *P. nigra* x *P. deltoides* hybrid with the same cultivar name produced by different subspecies of these two *Populus* species. Describing the true-to-type genotype for each

poplar cultivar has become cumbersome as (1) seedling trees are mislabeled as cultivars, (2) records of *P. nigra* and *P. deltoides* subspecies involved in a cross are lost, or (3) trees with unknown pedigree are identified as cultivars based on morphological traits. Rathmacher et al. (2009) found that poplar samples of the same cultivar differ in 2-4 bp, and that these small differences in the length of the repeats should be taken into account and be verify against a large number of samples considered as standards for each cultivar. Furthermore, to account for the high number of alleles per locus, Rathmacher et al (2009) suggested the construction of allelic ladders based on the multiple number of alleles observed for each SSR locus, and the creation of the allelic ladders should include as many standard samples and kwon cultivars as possible. With these allelic ladders, they were able to implement a more robust DNA-based fingerprint database for the certification and validation of poplar clones. Techniques used by Rathmacher et al (2009) could be applied to the creation of a cultivar validation program in chestnuts, as an allelic ladder could be created for the SSR loci used in this study. The allelic ladder based on chestnut reference genotypes will aid in the identification of chestnut species other than the usual morphological traits. Furthermore, with an allelic ladder for chestnut cultivars one could verify the high level of genetic diversity observed within 10 of the 11 cultivars included in this study.

Genotyping Cultivars

'Colossal': In the late 1990's and early 2000's, Fowler Nursery (Newcastle, CA) was the primary company selling grafted trees of the cultivar 'Colossal'. Many Michigan growers purchased cloned 'Colossal' trees from Fowler Nursery to establish their chestnut farms. Results from this study support the claim of Fowler Nursery that grafted 'Colossal' trees were genetically uniform, as I found one SSR profile for 'Colossal'. Furthermore, Fowler Nursery indicated to Michigan growers that all of their grafted 'Colossal' trees were produced by placing scion wood unto

rootstock of composed of 'Colossal' seedlings or 'Nevada' seedlings. Results from this study have demonstrated that 'Colossal' clones are genetically identical, and that all the seedlings used by Fowler Nursery as rootstock can be genetically differentiated from 'Colossal'. Gratifyingly, similar findings regarding ‘Colossal’ clonal character and ‘Colossal’ pollinizer cultivar differentiation were reported by McCleary et al (2013). In their study, McCleary et al (2013) used 11 EST-SSR markers to fingerprint 6 samples of ‘Colossal’ trees and 1 sample representing a cultivar commonly used as pollinizer of ‘Colossal’. The researchers described the high level of synonymy among the 6 samples of ‘Colossal’ included in their study, and they reported that ‘Colossal’ represents a single STRUCTURE group. Interestingly, the only sample included in their study and identified as ‘Colossal’ pollinizer belongs to a STUCTURE group that included true French (European x Japanese hybrids) cultivars. Results from this study regarding the association of the ‘Colossal’ pollinizer cultivars with French cultivars supports McCleary et al. (2013) findings. SSR makers, either genomic like SSR or genic like EST-SSR are useful to genetically differentiate the cultivars used as pollinizers of ‘Colossal’. Furthermore, results from my study supports McCleary et al (2013) hypothesis that SSR markers are useful tools for F1 progeny identification and the identification of the chestnut cultivars used as pollen donors (*i.e.* pollinizer trees) of ‘Colossal’.

'Benton Harbor': I expected to obtain the same level of uniformity in the SSR profiles for the samples within this Chinese hybrid since it has been clonally propagated by MSU researchers for about 10 years, yet surprisingly, the SSR profiles of the samples included as 'Benton Harbor' were not identical to each other as I had previously experienced with the cultivar 'Colossal'. The high level of diversity in grafts of 'Benton Harbor' and in multiple samples of leaf and bark samples from the 'Benton Harbor' ortet could be explained by several situations acting singly or

together. First, chestnut cultivars (*e.g*. 'Colossal') are known to develop seeds that divide into multiple embryos (Bassi and Craddock, 1998; Fulbright et al, 2010). It is possible that the 'Benton Harbor' ortet thought to be a single tree was a double embryo that fused during the development and maturity of the tree or the death of one of the two hypocotyl of the double embryo resulting in the single tree now standing as the 'Benton Harbor' ortet. Each embryo could have been produced by genetically different pollinizers (*i.e*. paternal parent) resulting in the multiple numbers of alleles seen in 'Benton Harbor' SSR profiles. Second, 'Benton Harbor' could be an example of a plant chimera. In general, a plant chimera could be the result of cellular mutations during the rapid cycles of mitosis, cellular division and tissue differentiation that occurs in the apical meristem of dicots (Pogany and Lineberger 1990; Lineberger 2012). In an adult chimeral plant, for example, the leaf and stem tissues could contain cells with different genotypes due to cellular mutations that occurred during embryogenesis, which were perpetuated through time. If a chimera produces non-phenotypic changes in a plant that is vegetatively propagated, as in grafted cultivars, it would be possible to miss the presence of the chimeral tissue in the "clones" of that plant. The diversity of grapevine varieties and its clonal variation due chimeras has been previously reported (review by Pelsy 2010; Hocquingny et al, 2004; Stenkamp et al, 2009). Furthermore, Franks et al. (2002) reported the usefulness of SSR profiling in the identification of chimeras in grapevine varieties. Third, the level of mutation rates experienced by SSR loci in chestnut might be in the order of 10^{-2} to 10^{-4}, which could be higher than the mutation rate anticipated occurring in plant genomes (described above). And lastly, the possibility of intrinsic mistakes made during the assignment of allele sizes of each SSR locus.

'Eaton': Among the cultivars included in this study, SSR profiling 'Eaton' samples represented the best example of homonymy, and perhaps, the problem of lacking a standard procedure to name

cultivars other than provenance. Nine 'Eaton' samples growing in Michigan orchards were thought to be grafted trees from the 'Eaton' ortets growing in Connecticut. However, it was suggested (*personal communication*, Dr. Sandra Anagnostakis) that the 'Eaton' grafted trees growing in Michigan were morphologically different than the 'Eaton' orterts growing in Connecticut, and that all 'Eaton' grafted trees growing in Michigan should be named 'Eaton River' based on the nursery where these trees were propagated (Nolin River Nut Tree Nursery, Kentucky). The 3 'Eaton' ortets from Connecticut consisted of 3 slightly different genotypes based on the presence of the 149 bp, 152 bp, and 155 bp alleles of CsCAT16. The SSR profile of 'Eaton River', thought to be a Chinese-Japanese-American hybrid like 'Eaton', was found to be genetically different from Connecticut's 'Eaton' ortets based on the presence of the unique 176 bp (CsCAT1) and 76 bp (EMCs15) alleles. Thus, 3 out of 9 'Eaton' samples (Eaton_BH, CK_Eaton R9T1, and Eaton 3/29/11) growing in Michigan appeared to be mislabeled because the SSR profiles contained the characteristic 176 bp (CsCAT1) and 76 bp (EMCs15) alleles. The remaining 6 'Eaton' samples growing in Michigan were not identical across all the alleles of the 5 SSR loci in the three SSR genotypes defined in this study as 'Eaton' ortets. One could suggest that there is a high level of diversity in grafted trees identified as 'Eaton' as in the case of grafted trees classified as 'Benton Harbor' (described above) or these trees are seedlings. The genetic variation observed in 'Eaton' could be an effect of high mutational rates for SSR loci in plants or mistakes performed during the assignment of alleles, or non-grafted trees. The genetic variation observed in ‘Eaton’ has been reported on the study conducted by McCleary et al. (2013). The researchers found that 6 out of 8 ‘Eaton’ samples included in their study were grouped with 2 of ‘Eaton Rapids’ and ‘Sleeping Giant’, which supports that ‘Eaton’ grafted trees are a descendent of ‘Sleeping Giant’. The remaining 2 ‘Eaton’ samples included in their study were grouped with

representatives of Chinese (*C. mollissima*) chestnut cultivars. Although this study consisted of only 1 sample of 'Eaton River' and the ortet was not included in order to clearly define the SSR profile of 'Eaton River' cultivar, interestingly, McCleary et al. (2013) commented on the high level of genetic diversity of the 'Eaton River' samples, which in their study 'Eaton River' samples (n=3) belong to two distinct STRUCTURE groups.

Genetic Relationship Among Chestnut Cultivars

I consider that the similarity depicted in the phenogram (Figure 3), where all individuals were genotyped as homozygotes consisting of two copies of the same allele, is an appropriate representation of the genetic relationship among the 114 chestnut samples included in this study, however it should not be considered to describe the taxonomic relationship among the cultivars. The similarities depicted in this phenogram follow the relationship among the cultivars based on the putative pedigrees previously described for most of the cultivars. For example, most of the cultivars considered as Chinese hybrids ('Benton Harbor' and 'Everfresh'), and 'Eaton' which is a cultivars that it is thought to be a hybrid between Chinese, Japanese, and American chestnut species were found in the same cluster. Furthermore, this phenogram (Figure 3) depicted the relationship between the three French chestnut cultivars, 'Bouche de Betizac', 'Mariguole', and 'Precose Miguole' (European x Japanese hybrids), as well 'J160' which is a cultivar with questionable Japanese pedigree into a single group, while the two European x Japanese propagated in California were grouped into a separate cluster. This observation is in agreement with previous findings regarding the genetic characterization and genetic relationship between 'Bouche de Betizac' and 'Colossal' (Boccacci et al, 2010). However, Boccacci et al (2010) observed a different genetic relationship among 'Mariguole', 'Precose Miguole' and 'Colossal' than what I reported herein. Boccacci et al (2010) indicated that based on 7 SSR loci 'Mariguole'

and 'Precose Miguole' belong to the same cluster as 'Colossal'. In this study, 'Mariguole' and 'Precose Miguole' were not clustered with 'Colossal'. The discrepancy of the genetic relationship among these three cultivars could have been an effect of the SSR loci used for SSR profiling. Boccacci et al (2010) used 7 SSR markers developed from *Castanea sativa* growing in Italy (*i.e*. CsCAT), while I used a total of 5 SSR markers, which included not only 3 of the CsCAT markers but also 1 SSR marker developed from *Castanea sativa* growing in the United Kingdom (*i.e*. EMCs) and 1 SSR marker developed from oak, *Quercus robur* (*i.e*. QrZAG). Regardless of the difference in the placement of 'Mariguole', 'Precose Miguole', and 'Colossal' into one or two clusters, it is interesting to see the level of resolution required of SSR profiling to separate cultivars with the same pedigree, that is, European x Japanese hybrids into unique clusters for each cultivar.

It becomes clear that more unique alleles of "new" SSR loci are needed to resolve closely related samples, as 'Colossal' grafted trees and the cultivars used as scion wood during the clonal propagation of 'Colossal'. Although, I expected to observe a higher level of differentiation between the European x Japanese hybrid, 'Colossal' and 'Okei' cultivar considered as Japanese-Chinquapin hybrid, the 5 SSR loci used for SSR profiling did not generate enough genotypic differences to observe a higher level of dissimilarity between these 2 cultivars in order to place these cultivars in 2 separate clusters. It is noteworthy to observe that 'Okei', 'Colossal', and 'Nevada samples represented a single cluster, which could be due to the propagation of these cultivars at Fowler Nursery in California.

Usefulness of SSR in the Identification of F1 progeny

The genetic identification of the F1 progeny (*i.e*. nuts) from the tri-parental cross ('Colossal', maternal parent, and 'Benton Harbor' and 'Okei' as paternal parents) was effectively accomplished with the SSR primers, EMCs15 and CsCAT1. All expected genotypes from this cross were observed, however the number of nuts with genotypes consisting of the 70 bp allele at the EMCs15 locus was low (70, 88; 1 out of 70 nuts, and 70,76; 3 out of 70 nuts). It is noteworthy, the low number of nuts with the 70 bp allele at the EMCs15 locus obtained from this tri-parental cross might be an indication of linkage disequilibrium or the effect of a non-favorable compatibility interactions between this 70 bp allele, inherited from the parent donor (*i.e.* pollen) and the allele inherited from the maternal donor ('Colossal'). Possible incompatible allelic interactions may be considered "lethal" or detrimental during the development of the embryo and nuts, causing a flat burs or empty cupulae. Studies testing the possible incompatible allelic interactions that may occur during pollination, fertilization or nut set among interspecific cultivars and the use of SSR as genetic markers of these incompatible interactions have not been conducted. Therefore, the reason for the low frequency of the 70 bp allele at the EMCs15 locus in this data remains uncertain. A much larger number of nuts (i.e sample size) than the number of nuts harvested for this experiment would be necessary to draw conclusions regarding the low frequency of the 70 bp allele.

The even distribution of the number of nuts with the remaining genotypes (not consisting of the 70 bp allele) favors random selection and assortment of alleles at the EMCs15 locus. Similar remarks cannot be drawn for the CsCAT1 locus because of the limited number of nuts that were genotyped with this SSR primer set. To my knowledge, this is the first report where SSR-fingerprinting is used to genetically identify the progeny of a cross among *Castanea* cultivars and hybrids in the United States. Although, in the last decade, scientists worldwide

have used SSR-based fingerprinting to genetically identify European, Chinese, and Japanese cultivars, and interspecific hybrids (Alvarez et al, 2005; Cheng and Huang 2009; Fernandez-Cruz and Fernadez-Lopez 2012; Han et al, 2007; Marinoni et al, 2013; Martin et al, 2005; Martin et al, 2011; Nishio et al, 2014). Recently, McCleary et al (2013) defined the interspecific pedigrees of 65 chestnuts cultivars currently grown in the United States based on 11 expressed sequence tag (EST)-SSR markers. They were able to verify the identity of 18 chestnut hybrids of European, Chinese, and Japanese origin. In their study, McCleary et al (2013) reported high levels of homonymy and synonymy within the 214 chestnut samples, and suggested that SSR-based genotyping is necessary for correct identification and geological ancestry of tree species like *Castanea*, which require cross-pollination for reproduction and nut-set.

Due to the low numbers of nuts harvested in this study and because the study was performed only during a single blooming season (2010), conclusions regarding which cultivar 'Benton Harbor' or 'Okei' is a better pollinizer of 'Colossal' female flowers cannot be drawn. The highest number of nuts (42 out of 70 nuts) were collected from the tri-parental cross performed in this study developed from 'Colossal' flowers exposed to airborne pollen on 12-July than from 'Colossal' flowers exposed to pollen from 23-June until 10-July. As expected, flowers exposed to pollen for a longer period of time during the blooming season produced more nuts than flowers exposed to pollen for a short period of time. Interestingly, flowers exposed to pollen mid-June to early-July (peak time of 'Colossal' female flowers receptivity, period determined to be the best time for pollination in the following experiments) did not resulted in the majority of the nuts collected from this study in 2010. It is possible that environmental conditions, such as cold and rain during mid-June to early-July in 2010 were not conducive to efficient pollination and/or dispersal of pollen. Garcia-Mozo et al (2004) conducted a survey of airborne pollen of

various tree species, which included *Castanea* spp., *Quercus* spp., and *Ulmus* spp., in La Mancha, Spain during two consecutive years, and the effect of temperature and rainfall on pollen dispersal. They found a positive correlation between temperature and pollen dispersal, as well as a negative correlation between rainfall and humidity with the abundance of pollen in the air.

Surprisingly, 'Colossal' flowers exposed to pollen through the entire blooming season (*i.e.* control) produced less nuts (14 out of 70 nuts) than those flowers exposed to airborne pollen only since 12-July (42 out of 70 nuts). A possible explanation for the low number of nuts that developed from the control, lower than those 'Colossal' flowers subjected to experimental conditions (*i.e*. placement and removal of bag on flowers), is the innate variability of chestnut production in the same orchard. It is gratifying to notice that covering flowers with bags to prevent pollination or removing bags on flowers to allow pollination did not interfere with nut-set or the normal growth and enlargement of the nuts.

The 'Okei' tree sired approximately twice as many 'Colossal' nuts (47 of 70 nuts) than the 'Benton Harbor' tree (22 of 70 nuts). Conclusions cannot be drawn as to which cultivar is the best pollinizer, and if the age or size of the tree had an effect in the number of nuts sired by each tree. Furthermore, it is difficult to conclude that the Japanese x Chinquapin cultivar ('Okei') has an advantage as pollinizer of 'Colossal' over the Chinese cultivar 'Benton Harbor' due to potential incompatible interactions between the Chinese cultivar and the European x Japanese cultivar 'Colossal' because these types of genetic interactions are currently under consideration. As scientists continue to elucidate the genetic interactions among *Castanea* species and robust methods of identification are applied to cultivars characterization, the role of the mother parent and pollen parent (*i.e*. pollen source) will become more evident. To date, various researchers (McKay and Crane 1939; Anagnostakis 1995; Rutter 1995) have reported that chestnuts species

are one of the few plant species that demonstrates the phenomena defined as xenia, where the male parent of a seed has an effect on the characteristics of the nut, such as size, weigh, color, nutrient content, flavor, and quality (Klinac et al, 1995). However, studies concerning the effect of pollen source on nut-set of interspecific hybrids has not been extensively investigated. In a study conducted by Hasegawa et al (2009), the genetic composition of Japanese chestnut (*C. crenata*) pollen grains and seeds was determined following SSR-fingerprinting. Hasegawa et al. (2009) determined that wild Japanese chestnut trees have the tendency to avoid self-pollination during nut-set, and that pollen source (*i.e*. male parent of a nut) must come from trees growing distant from the mother tree. Hasegawa et al (2009) provided the first study on the use of SSR markers to determine the pedigree of the nuts and its parents but because their study did not include hand, controlled pollination they were not able to determine the inheritance pattern of the seeds. Contrary to Hasegawa et al (2009), my study included a tri-parental cross ('Colossal', 'Benton Harbor' and 'Okei') of chestnut cultivars previously genotyped with SSR markers, controlled pollination in a single orchard, and the use of the bone marrow biopsy needle assay to extract DNA from kernels reducing the time of SSR genotyping (described below), which were all favorable conditions to determine the inheritance of the SSR alleles of each nut (*i.e*. F1 progeny).

Furthermore, to my knowledge, this is the first study where tissue from kernel (*i.e*. nuts) has been subjected to SSR-PCR amplification and the nut has been genotyped without disturbing the embryo to become a seedling. The bone marrow biopsy needle technique described in this study will allow researchers to sample kernel tissue from the nut immediately after the nut has been harvested. This sampling method (bone marrow biopsy needle assay) permits the rapid extraction of DNA from the nut, and ultimately reduces the time to genotype, characterize, and

identify the genotype of the nut (*i.e*. kernel). With such a fast assay, questions regarding pollination may be addressed without the need of leaf samples and the waiting time necessary for the nuts to develop into seedlings. In the event that DNA extraction from a kernel is unsuccessful, the researcher will have the opportunity to obtain a leaf sample from the nut with ease because the hole created by the needle and closing the hole in the nut with silicone will not interfere with the normal germination and development processes.

In summary, some unique alleles of the 5 SSR loci used in this study (namely CsCAT1, CsCAT2, CsCAT16, EMCs15, and QrZAG75) generated SSR profiles useful in the differentiation of 5 chestnut cultivars grown in Michigan; 'Colossal', 'Benton Harbor', 'Nevada', 'Okei', and 'Everfresh'. This study could potentially identify and describe unique features in Michigan's chestnut germplasm, which is predominantly composed of European x Japanese cultivars/hybrids, Chinese hybrids, and remnants of naturalized populations of American chestnuts. Results from this study will lead future investigation and implementation of genotyping programs necessary for the identification, validation and standardization of chestnut cultivars. Performing controlled crosses between cultivars with known pedigrees and unknown pedigrees in future studies may further enhance our understanding of chestnut pollination, the effect of pollen source (*i.e*. pollinizer trees) in an orchard based on genetic identification of the paternal trees and its progeny, and how genetic identification of chestnut cultivars and interspecific hybrids are necessary for a robust chestnut industry. Furthermore, results will assist growers in the establishment and maintenance of chestnut orchards because it could serve as a tool for the selection of chestnut cultivars with consistent horticultural characteristics and productivity based on genotypes.

LITERATURE CITED

LITERATURE CITED

Akkak, A., Boccacci, P., and D T., Marinoni. 2010. Cross-species amplification of microsatellite markers in *Castanea* spp. and other related species. Acta Hort. 866:195–201.

Aldrich, P R., Jagtap, M., Michler, C H., and J, Romero-Severson. 2003. Amplification of North American red oak microsatellite markers in European white oaks and Chinese chestnut Silvae Genetica 52:176–179.

Alvarez, J B., Martin, M A., Munoz, C., and L M, Martin. 2005. Genetic variability of chestnut in Andalusia (Spain). Acta Hort. 693:471–476.

Anagnostakis, S. L. 1995. Effect of the male parent on nut weight in chestnut. Annu. Rpt. Northern Nut Growers Assn. 86:124–127.

Anagnostakis, S L. 1999. Cultivars of Chestnuts, in: 90th Annual Report of the Northern Nut Growers Association, NNGA, Inc. Cornell, New York, USA.

Bassi, G., and H, Craddock. 1998. Poster number 35, Performance and description of the introduced chestnut cultivar 'Colossal' in Cuneo province, Norwest Italy in: 2nd International Chestnut Symposium (Abstract and List of Participants), Salesses, G. (ed.). ISHS, INRA.

Barakat, A., DiLoreto, D S., Zhang, Y., Smith, C., Baier, K., Powell W A., Wheeler, N., and J E, Carlson. 2009. Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to the chestnut blight infection. BMC Plant Biol. 9:51–62.

Beccaro, G L., Botta, R., Marinoni, D T., Akkak, A., and G, Bounous. 2005. Application and evaluation of morphological, phenological, and molecular techniques for the characterization of *Castanea sativa* Mill. cultivars. Acta Hort. 693:453–457.

Barreneche, T., Casasoli, M., Russell, K., Akkak, A., Meddour, H., Plomion, C., Villani, F., and A, Kremer. 2004. Comparative mapping between *Quercus* and *Castanea* using simple sequence repeats (SSRs). Theor. Appl. Genet. 108:558–566.

Boccacci, P., Canterino, S., Marinoni, D T., Mellano, M G., Beccaro, G L., and G, Bounous. 2010. Genetic characterization of chestnut accessions from 'Centro Regionale de Castanicoltura' (Cuneo, Italy) Acta Hort. 866:97–101.

Botta, R., Akkak, A., Guaraldo, P., and G, Bounous. 2005. Genetic characterization and nut quality of chestnut cultivars from Piedmonte (Italy). Acta Hort. 693:395–401.

Brookfield, J F Y. 1996. A simple new method for estimating null allele frequency from heterozygote deficiency. Mol. Ecol. 5:453–455.

Buck, E J., Hadonou, M., James, J., Blakesley, D., and K, Russel. 2003. Isolation and characterization of polymorphic microsatellites in European chestnut (*Castanea sativa* Mill.) Mol. Ecol. Notes 3:239–241.

Cantini, C., and A, Autino. 2009. Genetic characterization of Tuscan chestnut germplasm: genetic and genotypic variation among populations of three different areas. Acta Hort. 866: 233–238.

Cheng, L L., and W G, Huang. 2009. Analysis of genetic diversity among wild Chinese chestnut populations using SSR markers. ActaHort. 844:275–279.

Costa, R., Valdivieso, T., Marum, L., Borges, O., Soeiro, J., Assuncao, A., Fonseca, L., Matos Soares, F., Sequeira, J., and P, Correia. 2005. Characterisation of traditional Portuguese chestnut cultivars by nuclear SSRs. Acta Hort. 693:437–440.

Cuenca, B., Gonzalez, M V., Lopez, M., and M, Rey. 2010. SSRs molecular characterization of *Castanea sativa* Mill. genotypes selected for resistance to *Phytophthora cinnamomi*. Acta Hort. 866:181–187.

Fernandez-Cruz, J., and J, Fernandez-Lopez. 2012. Morphological, molecular, and statistical tools to identify *Castanea* species and their hybrids. Conservation Gen. 13:1589–1600.

Franks, T., Botta, R., and M R, Thomas. 2002. Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement. Theor. Appl. Gent. 104:192–199.

Fulbright, D W. 2011. Common Chestnut Cultivars Grown in Michigan in: Michigan State University's Rogers Reserve. Fulbright, D W (ed.) URL http://www.rogersreserve.org

Fulbright, D W. and M, Mandujano. 1999. Evaluation of the chestnut cultivar 'Colossal' in Michigan. Annu. Rep. Northern Nut Growers Assn. 90:48–55.

Fulbright, D W., Mandujano, M., and S, Stadt. 2010. Chestnut production in Michigan. Acta Hort. 866:531–537.

Garcia-Mozo, H., Perez-Badia, R., Fernandez-Gonzalez, F., and C, Galan. 2006. Airborne pollen sampling in Toledo, Central Spain. Aerobiologia. 22:55–66.

Graves, A.H. 1992. Illustrated guide of trees and shrubs:A handbook of the woody plants of the Northern United States and adjacent Canada. Dover Publications Inc. Mineola, New York.

Guo, X., and R C, Elston. 1999. Linkage information content of polymorphic genetic markers. Human Hered. 49:112–118.

Hamelin, R C., Bourassab, M., Rail, J., Dusabenyagasan, M., Jacobi, V., and G, Laflamme.

2000. PCR detection of *Gremmeniella abietina*, the causal agent of Scleroderris canker of pine. Mycol. Res. 104:527–532.

Han, J C., Wang, D J., Liu, Q X., and X Y, Zhang. 2007. Genetic diversity of Chinese chestnut (*Castanea mollissima*) in Hebei. Acta Hort. 760:575–577.

Hasegawa, Y., Suyama, Y., and K, Seiwa. 2009. Pollen donor composition during the early phases of reproduction revealed by DNA genotyping of pollen grains and seeds of *Castanea crenata*. New Phytologist 182: 994–1002.

Hocquigny, S., Pelsy, F., Dumas, V., Kindt, S., Heloir, M C., and D, Merdinoglu. 2004. Diversification within grapevine cultivars goes through chimeric states. Genome 47:579–589.

Jones, D A. 1972. Blood samples: power of discrimination. J. Forens. Sci. Soc. 12:355–359.

Kampfer, S., Lexer, C., Glossl, J., and H, Steinkellner. 1998. Characterization of (GA)n microsatellite loci from *Quercus robur*. Hereditas 129:183–186.

Kalinowski, S T., Taper, M L., and T C, Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16: 1099–1106.

Kelley, K.M., and B.K. Behe. 2002. Chef's perceptions and uses of 'Colossal' chestnuts. HortTechnology 12:172–175.

Kimura, M., and J F, Crow. 1964. The number of alleles that can be maintained in a finite population. Genetics 49:725–38.

Klinac, D J., Lelieveld, F., and R D, Knowles. 1995. Time of flowering and pollination in New Zealand chestnut selections, and introduced cultivars. New Zealand J. Crop Hort. Sci. 23:331–340.

Kubisiak, T L., and J H, Roberds. 2006. Genetic structure of American chestnut population based on neutral DNA analysis in: Restoration of American Chestnut To Forest Lands - Proceedings of a Conference and Workshop. Steiner, K C., and J E., Carlson, J. E., (eds.). The North Carolina Arboretum, Natural Resources Report, National Park Service. Washington DC, USA.

Lineberger, R D. 2012. Origin, Development and Propagation of Chimeras http://aggie-horticulture.tamu.edu/tisscult/chimeras/chimeralec/chimeras.html

Marinoni, D T., Akkak, A., Bounous, G., Edwards, K J., and R, Botta. 2003. Development and characterization of microsatellite markers in *Castanea sativa* (Mill.) Mol. Breed. 11:127–136.

Martin, M.A., Alvarez, J.B., Martin, L.M., Molina, J.R., Herrera, M.A., Mattioni, C., Cherubini, M., and F., Villani. 2011. Chestnut genetic landscape in Spain. Acta Hort. 918:843-847.

Martin, M A., Alvarez, J B., Mattioni, C., Cherubini, M., Villani, F., and L M, Martin. 2010a. Microsatellite-based characterization of traditional chestnut cultivars of Italy. Acta Hort. 866:157–162.

Martin, A C., Gimenez, M J., and J B, Alvarez. 2005. Varietal Identification of Chestnut Using Microsatellites Markers. Acta Hort. 693:441-446.Martin, A C., Gimenez, M J., and J B, Alvarez. 2004. Varietal identification of chestnut using microsatellite markers. Acta Hort. 693:441–452.

Martin, M A., Mattioni, C., Cherubini, M., Taurchini, D., and F, Villani. 2010b. Genetic characterisation of traditional chestnut varieties in Italy using microsatellites (simple sequence repeats) markers. Ann. Appl. Biol. 157:37–44.

Martin, M A., Mattioni, C., Cherubini, M., Taurchini, D., and F, Villani. 2010c. Genetic diversity in European chestnut populations by means of genomic and genic microsatellite markers. Tree Genet. Gen. 6:735–744.

Mattioni, C., Cherubini, D., Villani, F., and M A, Martin. 2010. Evaluation of genomic SSRs and EST-SSRs markers in genetic diversity studies in European chestnut populations. Acta Hort. 866:151–156.

Mattioni, C., M A, Martin, M A., Cherubini, D., Taurchini, D., and F, Villani, F. 2010. Genetic diversity in European chestnut populations. ActaHort. 866:163–167.

Mattioni, C., Martin, M A., Pollegioni, P., Cherubini, M., and F, Villani. 2013. Microsattelite markers reveal a strong geographical structure in European populations of *Castanea sativa* (*Fagaceae*): evidence for multiple glacial refugia. Am. J. of Botany 100: 951–961.

McCleary, T., McAllister, M., Coggeshall, M., and, J. Romero-Severson. 2013. EST-SSR markers reveal synonymies, homonymies and relationships inconsistent with putative pedigrees in chestnut cultivars. Genet. Resour. Crop Evol. 60:1209–1222.

McDonald, D. 2008. Molecular Marker Glossary. www.uwyo.edu/dbmcd/molmark/mcdgloss.html

McKay, J W., and H L, Crane. 1939. Xenia in the chestnut. Science 89:348–349.

Medina-Mora, C M. 1999. Genetic variation in strains of *Clavibacter michiganensis* subsp. *michiganensis* and the development of bird's eye fruit lesions on tomatoes. Thesis (M.S.), Michigan State University. Dept. of Botany and Plant Pathology, East Lansing, Michigan.

Medina, C M., and D W, Fulbright. 2010. Evaluation of simple sequence repeats (SSR) for genetic analysis of chestnut trees in Michigan orchards. Acta Hort. 866:127–133.

Miller, G. 2003. Chestnuts, in: A guide to nut tree culture in North America. Fulbright, D (ed.). Volume 1. Northern Nut Growers Assn., Inc. Cornell, New York.

Nei, M. 1972. Genetic distance between populations. Amer. Naturalist 106:283–292.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA 70:3321–3323.

Nishio, S., Iketani, H., Fujii, H., Yamamoto, T., Terakami, S., Takada, N., and T, Saito. 2014. Use of population structure and parentage analyses to elucidate the spread of native cultivars of Japanese chestnut. Tree Gen. Genom. 10:1171–1180.

Nishio, S., Yamamoto, T., Terekami, S., Sawamura Y., Takada, N., and T, Saito. 2011. Genetic diversity of Japanese chestnut cultivars assessed by SSR markers. Breed. Sci. 61:109–120.

Ohata, T., and T., Sato. 1961. Studies on the pollination of chestnut trees. Bulletin Nat. Inst. Agri. Sci. Ser E 9:185–193.

Paetkau, D., Calvert, W., Stirling, I., and C, Strobeck. 1995. Microsatellite analysis of population structure in Canadian polar bears. Mol. Ecol. 4: 347–354.

Pelsy, F. 2010. Molecular and cellular mechanisms of diversity within grapevine varieties. Heredity 104:331–340.

Pogany, M F., and R D, Lineberger. 1990. Phenotypic variation during micropropagation of the chimeral *Rhododendron* 'President Roosevelt'. Plant Cell, Tissue and Organ Culture 21:201–220.

Powell, W., Machray, G C., and J, Povan. 1996. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. 1:215–222.

Rathmacher, G., Niggemann, M., Wypukol, H., Gebhardt, K., Ziegenhagen, B., and R, Bialozyt. 2009. Allelic ladders and reference genotypes for a rigorous standardization of poplar microsatellite data. Trees 23:573–583.

Rohlf, F J. 1998. Department of Ecology and Evolution, State University of New York, Stony Brook, New York.

Rutter, P A. 1996. Chestnut pollinator's guide. Bulletin 1, Badgersett Research Corporation, Pensylvania, USA.

Sisco, P H., Casasoli M., Berreneche T., Kremer, A., Hebard, F V., Kubisiak, T L., Clark, C., Sederoff, R R., and F, Villani. 2005. An improved genetic map for *Castanea mollissima*

/*Castanea dentata* and its relationship to the genetic map of *Castanea sativa*. Acta Hort. 693:491–495.

Stenkamp, S H G., Becker, M S., Hill, B H E., Blaich, R., and A, Forneck. 2009. Clonal variation and stability assay of chimeric Pinot Meunier (*Vitis vinifera* L.) and descending sports. Euphytica 165:197–209.

Summers, K., and W, Amos. 1997. Behavioral, ecological and molecular genetic analyses of reproductive strategies in the Amazonian dart-poison from, *Dentrobates ventrimaculatus*. Behav. Ecol. 8: 260–267.

The Census of Agriculture. 2007; Available from: http://www.agcensus.usda.gov/Publications/2007/Full_Report/Volume_1,_Chapter_1_State_Level/Michigan/index.asp. USDA.

Thuillet, A C., Bru, D., David, J., Roumet, P., Santoni, S., Sourdille, P., and T, Bataillon. 2002. Direct Estimation of Mutation Rate for 10 Microsatellite Loci in Durum Wheat, *Triticum turgidum* (L.) Thell. ssp *durum* desf. Mol. Biol. Evol. 19:122–125.

Udupa, S M., and M, Baum. 2001. High mutation rate and mutational bias at (TAA)n microsatellite loci in chickpea (*Cicer arietinum* L.). Mol. Genet. Genomics 265:1097–1103.

Wagner, H W., and K M, Sefc. 1999. IDENTITY 1.0. Centre for Applied Genetics, University of Agricultural Sciences, Vienna.

Wang, Y., Kang, M., and H W, Haung. 2008. Microsatellite loci transferability in chestnut. J. Am. Soc. Hort. Sci. 133:692–700.

Weir, B S. 1996. Genetic Data Analysis II. Sinauer Associates, Inc. Publishers Sunderland, Massachusetts.

Yamamoto, T., Kotobuki, K., Matsuta, N., Suzuki, M., and T, Hayashi. 2003. Characterization of simple sequence repeats in Japanese chestnut. J. Hort. Sci. Biotech. 78:197–203.

Yeh, F C., Yang R., and T, Boyle. 1999. Microsoft Window-based freeware for population genetics analysis. University of Alberta and Centre for International Forestry Research, Alberta, Canada.

STUDIES TO DETERMINE PHENOLOGY AND POLLINATION IN THE EUROPEAN X JAPANESE HYBRID ‘COLOSSAL’ IN MICHIGAN ORCHARDS

ABSTRACT

Chestnut trees (*Castanea* spp.) are monoecius and require cross-pollination for successful nut set. Pollination is dependent on the synchronization between female flowers and pollen dehiscence, the quantity and quality of mature pollen grains, and climatic conditions favorable for flower and pollen interaction. Nut production may decrease in chestnut forests and orchards when flowering and pollen dispersal are not in synchrony, yet very little is known about the phenology of chestnut and the timing of pollination in Michigan-grown chestnuts. The purpose of this study was to improve our understanding of chestnut phenology, pollination, and its relation to nut set (yield) by conducting both natural, and hand-pollination experiments. In these experiments, cv. 'Colossal', a European X Japanese cultivar was selected as the mother tree due to the large number of trees planted during the late 1990s and 2000s and because of its yield advantage over other chestnut cultivars planted in Michigan orchards.

Natural pollination in the cultivar 'Colossal' was monitored for three years (2008, 2009, and 2010) and at two orchard sites located in mid-Michigan (Eaton Rapids and Clarksville). The cultivars 'Okei', a Japanese X Allegheny chinquapin (*C. crenata* X *C. pumila* var. *pumila*) hybrid, and 'Benton Harbor', a Chinese (*C. mollissima*) seedling selection were used as pollinizers of ‘Colossal’. Pollination was permitted or inhibited by placing bags over flowers or by removing bags from flowers at preset times. Pollination of ‘Colossal’ female flowers was initiated on 2-July (2008), 25-June (2009), and 18-June (2010). The highest number of nuts resulted when ‘Colossal’ flowers were continuously exposed to pollen until 22-July (2008), 5-July (2009), and 2-July (2010).

To perform hand pollination experiments with 'Benton Harbor' or ‘Okei’ pollen, a single catkin was removed from the paternal trees and rubbed onto the stigmata of 'Colossal' female flowers. Prior to and after hand application of pollen, flowers were protected from unwanted pollen by placing bags over the flowers. In 2009 and 2010, hand pollination experiments were conducted in orchards located in East Lansing and Clarksville, MI. ‘Colossal’ female flowers were pollinated once, twice or three times at pre-anthesis, anthesis and post-anthesis flower development stages in 2009 and 2010, ‘Colossal’ female flowers on trees at Clarksville, MI were pollinized once, twice, three or four times at the same developmental stages with a fourth, late post-anthesis, added to the study in 2009. Results suggested that pollen applied just a single time, presumably at the time of ‘Colossal’, anthesis, provided greater nut set than when pollen was applied a single time earlier or later. Flowers exposed to pollen on 28-August did not produce any nuts.

Results from the natural-pollination and hand-pollination experiments strongly suggest that the highest number of ‘Colossal’ nuts is achieved when pollen is available at anthesis. Anthesis in mid-Michigan is generally between late-June and early-July, regardless of the year, and that the specific dates for optimal pollination fluctuates slightly from year to year.

INTRODUCTION

Pollination is the method by which pollen grains dehisce from pollen sacs within the anthers and are transferred to the stigma of angiosperms by either insects (*i.e*. entomophily) or wind (*i.e*. anemophily) (Akerman, 2000). All *Castanea* species are anemophilus trees and monoecius, where unisexual and bisexual flowers are borne on a single tree (Miller, 2003). Pollination in *Castanea* species takes place after anthesis of unisexual staminate flowers (*i.e*.

male catkins). In *Castanea* species, xenogamy (*i.e*. allogamy) occurs when the ovules of one plant are fertilized by the pollen of another plant, which implies cross-pollination. Xenogamy in chestnuts is required for nut set, as all *Castanea* species cross-pollinate or hybrize. Xenogamy is essential for all fruit trees when the pollen on a tree is self-sterile or self-incompatible with the stigma as with *Castanea* species. Once these conditions are met, *Castanea* species will spontaneously hybridize, and will produce viable and fertile hybrid seedlings when pollen grains of one species or cultivar efficiently fertilize the ovules on maternal trees.

Multiple factors affect pollination of fruit-bearing trees in temperate regions. In general, factors include climatic conditions, phenology of pollen dehiscence, and duration of female blooming time (Dinis et al, 2010), as well as orchard composition (Nyeki, 1996). Windy, dry, and warm temperatures are usually favorable climatic conditions for pollen dehiscence, pollen transfer, and pollen adhesion to stigmatic surfaces. The time of pollen dehiscence as well as the quantity of pollen grains released to the atmosphere varies among chestnut species and cultivars. Mert and Soylu (2006) determined that the number of pollen grains in an anther varied from 3,850 to 5, 200 among male-fertile *C. sativa* cultivars. Although the blooming time of chestnuts can be as long as 30 days (Soltesz, 1996), only a limited amount of research has been conducted to precisely estimate the peak time of female flower receptivity because this parameter greatly depends on the cultivars' genetic composition, the cultivar geographic origin, and the onset of vegetative growth under the unique climatic pressure experienced at each planting in any given year (Costa et al, 2008; Fulbright et al, 2010; Soltesz, 1996). Research has established that female flowers on chestnuts maintain a long pollination period of over 20 days, but the optimum pollination time is between 11 and 15 days after stigmata emerged from female flowers (Shi et al, 2003), or when styles are fully extended and within 3 to 5 days after styles stopped growing

(Nakamura, 1992). According to Nakamura (2001), the pollination process culminates in fertilization 24 days after pollen adheres to the stigmatic surfaces on *C. crenata* flowers. Furthermore, Fulbright and Mandujano (2001) found that effective pollination, fertilization, and nut set in four-year-old chestnut orchards occurred when potential pollinizer trees were planted in close proximity (within 7.5 meters) to maternal trees.

In newly established orchards in Michigan, empty cupules (*i.e*. burs) are common and on average less than one nut per bur is harvested (Fulbright and Mandujano, 2001). Michigan is not the only state experiencing low yields. Previous studies demonstrated that well-established Chinese chestnut orchards experienced a large percentage of empty burs, ranging from 10-90% (Bai, 1988; Hunt et al, 2005; Shi and Stosser, 2005; Wang et al, 2001). Researchers suggested that the occurrence of empty burs appears to involve poor pollination and inadequate fertilization, which occur prior to nut set. In Michigan orchards, flower production is not the problem as by the third growing season, some cultivars may typically have over a hundred female flowers available for pollination and nut set (*personal communication*, Dr. Dennis Fulbright). Anecdotal observations of pollination in Michigan orchards, have lead researchers to the hypothesis that pollination can be problematic and that limited amounts of pollen from young trees, improper timing of dehiscence, and/or pollen-stigma incompatibility may be playing significant factors leading to the lack of nut set. Therefore, the main objective of this study was to improve our understanding of chestnut reproductive phenology, pollination, and nut set. This was accomplished by conducting natural-controlled pollination and controlled hand-pollination experiments. Results from this study will help determine how long 'Colossal' female flowers are receptive under mid-Michigan's climatic conditions and if timing of pollen maturity and dehiscence is a crucial factor in nut-set. Furthermore, this knowledge will help Michigan's

chestnut industry find alternative pollinizers for 'Colossal' trees in order to reduce yield loses due to empty burs.

MATERIALS AND METHODS

Chestnut Tree species and Cultivars

The cultivar known as 'Colossal' is considered a European x Japanese (*Castanea sativa* x *C. crenata*) hybrid tree (Anagnostakis, 1999) propagated by Fowler Nursery in Newcastle, California (Table 10). In this study, 'Colossal' was selected as the mother tree because the majority of Michigan orchards contain this cultivar not only because of the large number of trees planted during the late 1990s and early 2000s but also because of its tremendous yield advantage over any other chestnut cultivar planted in Michigan (Fulbright and Mandujano, 1999). Like all chestnut trees, 'Colossal' trees require cross-pollination for nut production. Trees of the cultivars known as 'Okei' and 'Benton Harbor' were selected as pollinizers. 'Okei' is a Japanese x Allegheny chinquapin (*C. crenata* x *C. pumila* var. *pumila*) hybrid thought to be a seedling of 'Silverleaf', a cultivar propagated in California with predominant characteristics of Japanese germplasm (Anagnostakis, 1999). While, 'Benton Harbor' trees are grafted trees selected by researchers at Michigan State University. The 'Benton Harbor' ortet tree was selected from open-pollinated Chinese (*C. mollissima*) seedlings trees planted at the Southwest Michigan Research and Extension Center (SWMREC) in Berrien County (Benton Harbor), Michigan, by a group of volunteers from the Midwest Nut Producers Council (Table 10).

Natural Pollination

Natural pollination in the European x Japanese cultivar 'Colossal' was monitored for three years. In 2008 and 2009, an isolated 4-year-old chestnut planting located in Eaton County (Eaton

Table 10. Name of the chestnut cultivars and type of inflorescence used in this study, and the putative *Castanea* species each cultivar represents.

Cultivar	**Type of inflorescence**	**Hybrid**	**Putative Species** [a]
'Colossal'	female	European-Japanese	*Castanea sativa* x *C. crenata*
'Benton Harbor'	male (catkin)	Chinese selection	*Castanea mollissima* hybrid
'Okei'	male (catkin)	Japanese-Allegheny chinquapin	*Castanea crenata* x *C. pumila* var. *pumila*

a *Castanea* species suggested to be involved in the cross from which the mother tree was selected to produce grafted trees of each cultivar (Anagnostakis, 1999; Fulbright, *personal communication*).

Rapids), Michigan was used as the study site. In 2010, the study site was moved to another isolated 4-year-old chestnut planting located in Ionia County (Clarksville), Michigan. Both chestnut plantings had similar layouts and consisted of two parallel rows of chestnut trees. The trees were planted in 2002 following a design of 7.5 X 7.5 meters. Six 'Colossal' trees at each site served as maternal parents and were planted in a single row downwind (east) from a row of pollinizer trees. All pollinizer trees were male-fertile and pollen grains dehisced during or close to the blooming time of 'Colossal' female flowers. This design allows up to three cultivars at each location to serve as pollen sources for the maternal trees. When only one pollen source was used, the other cultivars were emasculated by removing catkins prior to pollen maturity and removing the catkins from the site. At the Eaton Rapids planting, a single, 'Okei' tree, 10 m in height, was the only pollinizer allowed to produce pollen in 2008 and 2009. At the Clarksville chestnut planting, two 'Benton Harbor' trees 3 meters in height served as pollen sources in 2010. In 2008, bags were placed on 'Colossal' flowers on 18-June, 26-June, 3-July, 11-July and 23-July. In 2009, bags were placed on 'Colossal' flowers on 26-June, 30-June, 6-July, and 3-August. In 2010, bags were placed on 'Colossal' flowers on 9-June, 19-June, 26-June, and 3-July.

Natural Pollination—covering of flowers with bags at various dates

Natural pollination between 'Okei' pollen and 'Colossal' bisexual inflorescences was allowed to occur by natural means (air currents). To block pollination, 'Colossal' flowers were covered with white corn pollination bags (Lawson #451, Lawson Pollinating Bags, Northfield, IL) at various dates during pollen dehiscence. Bags were securely placed over flowering branches following the suggestions described by Rutter (1996). Each 'Colossal' bisexual inflorescence consisted of 1-3 female flowers with three groups of 7-9 styles (Figure 4). A total of 120, 296, and 240 flowers per treatment date were included in this study during 2008, 2009,

A

B

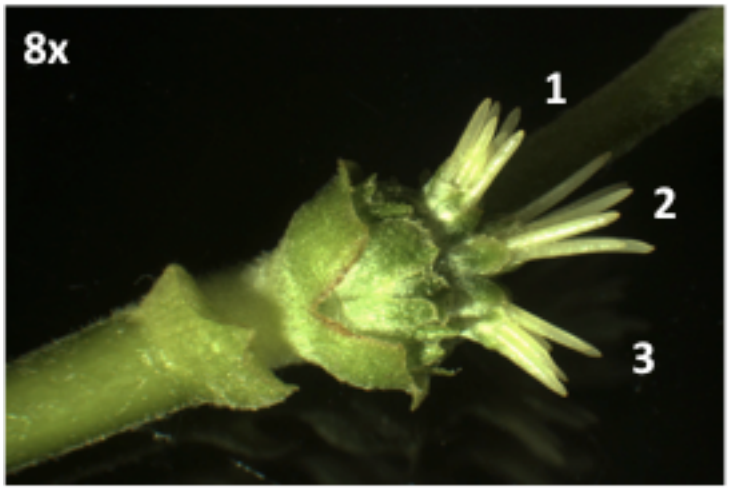


Figure 4. Bisexual catkins on 'Colossal' trees contained 1-3 female flowers (A). Insert on panel A represents the developmental stage of female inflorescences or flowers on 'Colossal' trees at the time of pollination. Each female flower (B) consisted of three groups of 7-9 styles (1, 2, and 3). (1x and 8x magnification for panels A and B, respectively)

and 2010, respectively. All female flowers remained covered with pollination bags until replaced with fresh mesh produce bags (5-10 lbs. #PB-507, Monte Package Company, Riverside, MI) in late August of each year, to aid in collection of burs and nuts at harvest.

Natural Pollination—uncovering flowers by removal of bags at various dates

Also, in 2009, a total of 600 flowering branches were covered with pollination bags before the florets of the 'Okei' catkins opened. To determine that all 'Okei' pollen catkins were closed on 20-June, 10 catkins were removed from the 'Okei' tree and were observed under the dissecting microscope. One hundred bags were removed on 26-June, 30-June, 6-July, 3-August, and 28-August, where flowers were exposed to 'Okei' pollen. Flowers remained exposed to 'Okei' pollen until small burs were observed. In addition to these flowers, another 25 flowering branches remained covered for the season. Burs were covered with fresh produce mesh bags (5-10 lbs. #PB-507, Monte Package Company, Riverside, MI) in late August, to aid in the collection of burs and nuts at harvest.

Burs were harvested on 7-October, 9-October, and 21-September in 2008, 2009, and 2010, respectively. The total number of burs, the number of nuts, the position of nut within each bur, and the weight of each nut were recorded. The total number of nuts would be reported in this dissertation.

Hand Pollination

'Colossal' pistillate flowers were hand-pollinated during the 2009 and 2010 growing seasons at a 4-year-old chestnut planting located in Ingham County (East Lansing), Michigan. This planting was similar to those described above and consisted of two rows of chestnut trees, where trees were planted in 2002 following a design of 7.5 X 7.5 meters. All 4 'Colossal' trees were planted in a single row downwind from pollinizer trees. The pollinizer trees were emasculated each year

since they were not needed for this study. In 2009, 'Colossal' trees at the Clarksville planting were also included to this study to increase the number of flowers pollinated.

For pollen application at the East Lansing planting in 2009 and 2010, fresh unisexual mesostaminate catkins (Figure 5) from 'Benton Harbor' trees were manually detached from flowering branches at the catkin's peduncle. 'Benton Harbor' catkins were collected either 24-hours prior to pollen application date or on the date of pollen application. Catkins collected 24-hours prior to application date, were stored at 4º C until application time. On the day of pollen application, 'Benton Harbor' catkins were stored and taken to the field in plastic bags (quart-size) inside an ice-containing, thermal-insulated cooler.

In 2009, for pollen application at the Clarksville orchard, fresh unisexual longistaminate catkins (Figure 5) from cultivar 'Okei' trees were detached from flowering branches as described above. Catkins were dried for 24 hours at room temperature and stored inside aluminum foil containing a cheesecloth pouch filled with pellets of calcium carbonate. Dry catkins were used when fresh catkins were not available during pollen application.

Hand Pollination—three different flower development stages

To perform hand pollination with 'Benton Harbor' pollen, a single catkin was rubbed onto the stigmata of one to five 'Colossal' flowers on one flowering branch. Immediately after each pollen application, the catkin was taped to the flowering branch in proximity to the 'Colossal' bisexual inflorescences. Each individual flowering branch was immediately covered with a pollination bag to prevent stray pollen from accidentally pollinizing the flowers. A total of 118 and 120 flowering branches per pollen application date were bagged in 2009 and 2010, respectively. Female flowers were pollinated once, twice or three times. In 2009, pollen was applied on flowers' stigmata on 26-June, 3-July, and 9-July three times. In 2010, pollen was

A

B

C

D

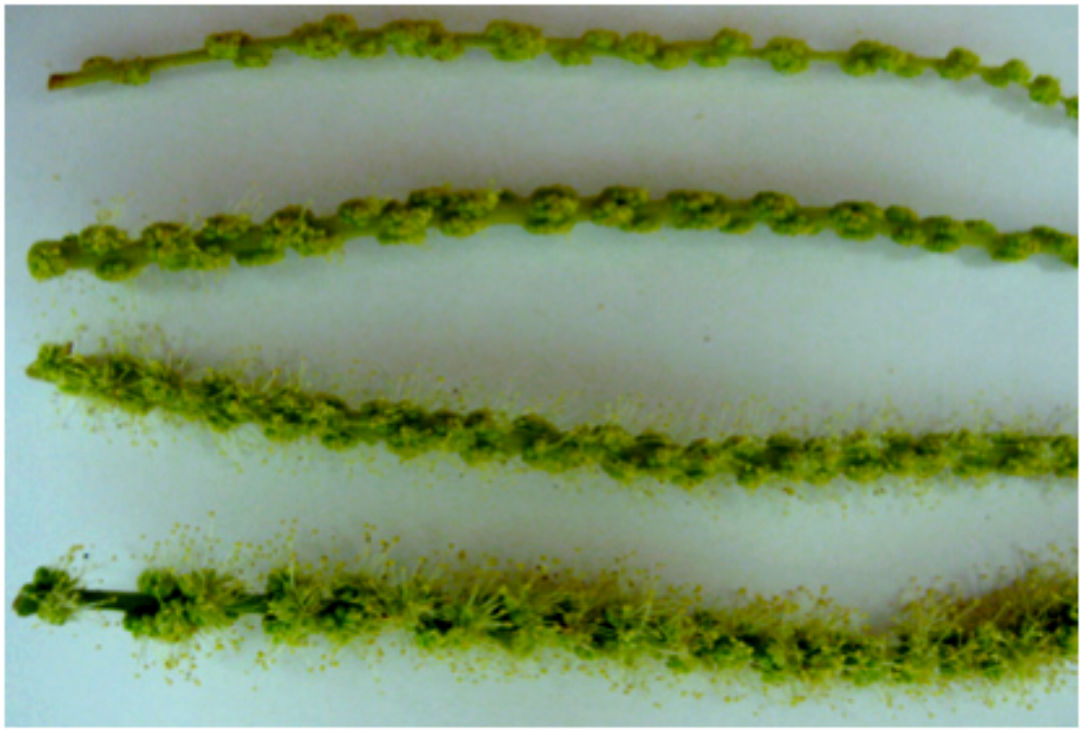


Figure 5. Morphology of chestnut catkin based on stamens length and pollen fertility. Astaminate catkin (A) consists of sterile male florets with non-functional pollen. All catkins with fully functional pollen are classified as: brachystaminate (B), mesostaminate (C), and longistaminate (D). Chestnut cultivars representing astaminate (A), mesostaminate (C), and longistaminate (D) catkins are 'Colossal', 'Benton Harbor', and 'Okei', respectively. (1x magnification)

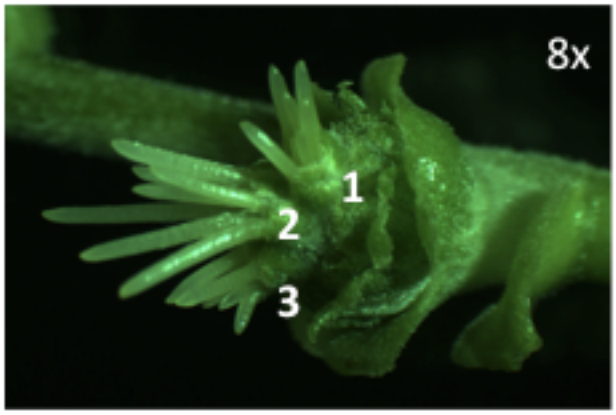
applied on flowers' stigmata on 17-June, 24-June, and 6-July. On these three application dates, 'Colossal' female flowers were considered to be at pre-anthesis, anthesis, and post-anthesis stages (Figure 6). The flowering period of 'Colossal' female flowers was defined by differences in the morphology of its inflorescences. Each consisted of three female flowers, where each female flower or gynoecium represented the pistil, which it was made up by the ovary, the placenta, the ovules, the styles and the stigma. Each pistillate flower had three groups of 7-9 styles, two lateral groups and one central group (Figure 4B). Flowers determined to be at pre-anthesis consisted of inflorescences where the central group of styles was approximately 2 mm longer than the two lateral groups. Flowers determined to be at anthesis consisted of inflorescences where the central group of styles and the two lateral groups of styles were fully extended and all three groups were approximately 7mm in length. Flowers determined to be at post-anthesis consisted of inflorescences where the central and two lateral groups had the same length as the flowers at anthesis, however the styles of the flowers had a curvature at the distal end of the styles and the soft spines of immature burs were noticeable (Figure 6).

Hand Pollination—four different flower development stages

In 2009, at another chestnut planting, 'Colossal' flowers were hand pollinated once, twice, three or four time to repeat the experiment described above and to determine if a late pollen application, 4-August, would have an effect on 'Colossal' nut production. Ninety 'Colossal' flowers were pollinized with ‘Okei’ pollen on 23-June, 2-July, 7-July, and 4-August.

Burs were harvested on 8-October and 22-September in 2009 and 2010, respectively. The total number of burs, the number of nuts, the position of nut within each bur, and the weight of each nut were recorded. Nuts were collected on 10-October and 25- September in 2009 and 2010, respectively. The number of nuts will be reported in this dissertation.

A



B

C

Figure 6. Morphology of 'Colossal' female flowers representing the developmental stages of the flowers selected for controlled hand-pollination. Panels A-C represent flowers at pre-anthesis, anthesis, and post-anthesis stages, respectively. Central group of styles (2) was approximately 2 mm longer than the lateral groups of styles (1 and 3) when flowers were at pre-anthesis stage (A). Flowers at anthesis had the central group and the lateral groups of styles fully extended and all three groups of styles had the same length of approximately 7 mm (B). Through time, the length of the styles of flowers at post-anthesis had the same length of the styles at anthesis, however the styles of flowers at post-anthesis had a curvature at the distal end and the soft spines of immature burs were noticeable (C). (8x, 2x, and 1x magnification for panels A, B, and C, respectively).

RESULTS

Natural Pollination

To determine when 'Colossal' flowers are receptive to pollen, I monitored the natural pollination of 'Colossal' flowers during the 2008, 2009, and 2010 seasons by recording nut production obtained on various dates when pollination was allowed or blocked through the process of removing or placing bags on the flowers.

Natural Pollination—placement of bags on flowers at various dates

To determine when pollination was initiated and when peak pollination was observed in each of three years, flowers were exposed to pollen until a preset date when pollen was blocked by placing a paper bag over the flowers. The flowers could only be pollinized if pollen was available prior to the placement of the bags. By recording the number of nuts obtained from the flowers blocked to pollen on specific dates, I was able to determine the approximate date of the onset of pollination.

In 2008, flowers that were exposed to possible pollination until 25-June and then covered with a bag to prevent possible pollination from that date on produced no nuts indicating that pollination had not occurred by 25-June of that year. Flowers that were exposed until 2-July and then covered with a bag to prevent possible pollination from that date on produced 4 nuts indicating that pollination had started sometime between 25-June and 2-July. Flowers that were exposed until 10-July and then covered with a bag to prevent possible pollination from that date on produced 17 nuts indicating that pollination had occurred at some period prior to10-July. Flowers that were exposed until 23-July and then covered with a bag to prevent possible pollination from that date on produced 20 nuts indicating that pollination had occurred at some period prior to 23-July (Table 11 and Figure 7). These results suggest that pollination started

Table 11. Effect of pollination time on the total numbers of nuts collected from European x Japanese chestnut trees cv. 'Colossal' growing in plots located in Eaton Rapids (Eaton County), MI (2008 and 2009) and Clarksville (Ionia County), MI (2010).

Treatment—Bag placed on flower [a] (date)			Total number of nuts [b] (date)		
2008	**2009**	**2010**	**2008**	**2009**	**2010**
17-Jun	**25-Jun**	**8-Jun**	0	8	0
25-Jun	**29-Jun**	**18-Jun**	0	50	3
2-Jul	**5-Jul**	**25-Jun**	4	64	73
10-Jul	**2-Aug**	**2-Jul**	17	45	143
22-Jul	**__ c**	—	20	—	—

a Dates on which 60, 148, and 120 'Colossal' female flowers were covered with pollination bags (Lawson #451) during the 2008-2010 seasons, respectively. Pollination bag placed on 'Colossal' female flowers prevents pollination from the date of its placement over the flowers.

b Number of nuts collected during the 2008-2009 seasons. Burs were harvested on 7-October (2008), 9-October (2009), and 21-September (2010).

c The sign (—) represents data not included.

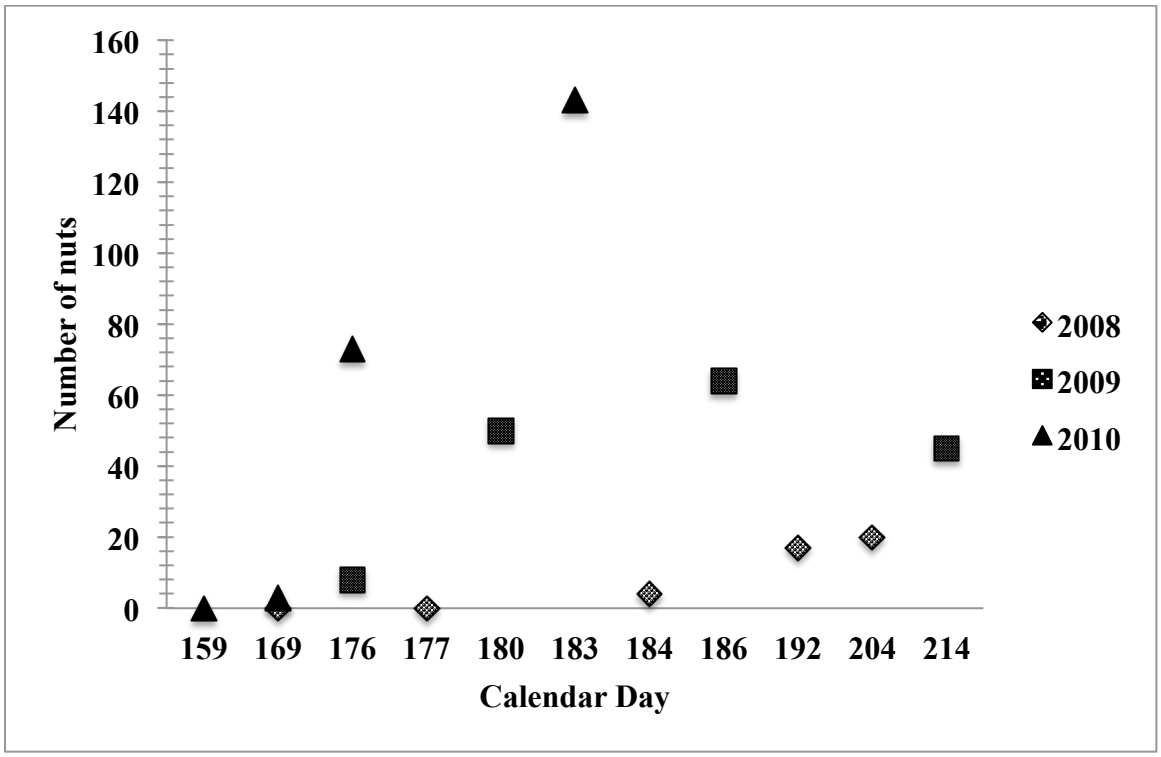


Figure 7. The number of nuts produced by flowers of European x Japanese chestnut cv. 'Colossal' when pollination was allowed to occur until a preset date when pollination was blocked by placing a bag over the flower after the date indicated. Nuts were collected from trees in plots located in Eaton Rapids, MI (2008 and 2009) and Clarksville, MI (2010). Calendar day represents the actual day when bags were placed over flowers. Day 159 = 8-June and day 214 = 2-August. In 2008, nuts were first recorded on day 184 (2-July); in 2009, nuts were first recorded on day 176 (25-June); and in 2010, on day 169 (18-June).

sometime after 25-June and increased slightly during the summer. The observed trend suggested the longer the exposure to possible pollen, the larger the nut set. This experiment was repeated in 2009.

In 2009, flowers that were exposed to possible pollination until 25-June and then covered with a bag to prevent possible pollination from that date on produced 8 nuts indicating that pollination in 2009 had started by 25-June of that year; earlier than 2008. Flowers that were exposed to possible pollination until 29-June and then covered with a bag to prevent possible pollination from that date on produced 50 nuts indicating that pollination had substantially increased during the 4 day period after 25-June and before 29-June (Table 11 and Figure 7). Flowers that were exposed to possible pollination until 5-July and then covered with a bag to prevent possible pollination from that date on produced 64 nuts indicating that pollination had occurred prior to 5-July. That 64 nuts were recorded 6 days after 50 nuts had been recorded suggests that pollination had continued to increase but that cannot be substantiated by these data. Flowers that were exposed to possible pollination until 2-August and then covered with a bag to prevent possible pollination from that date on produced 45 nuts indicating that pollination had occurred after 25-June and before 2-August (Table 11 and Figure 7). Because the number of nuts is lower than the previous dates (50 and 64 nuts, 29-June and 5-July, respectively), and nuts cannot disappear, these data suggest there is a high variability in the number of nuts produced per date per set of flowers monitored. However, the order of magnitude does not change and a trend toward longer exposure to pollen the larger nut set is observed in both 2008 and 2009. The important data from 2008 and 2009 is that pollination did not start on the same date and that nut set was greater in 2009 than 2008 (Table 11 and Figure 7). This experiment was repeated in 2010 using earlier dates than 2008 and 2009.

In 2010, flowers that were exposed to possible pollination until 8-June and then covered with a bag to prevent possible pollination from that date on produced no nuts indicating that pollination in 2010 had not started by 8-June of that year. Flowers that were exposed to possible pollination until 18-June and then covered with a bag to prevent possible pollination from that date on produced 3 nuts indicating that pollination had started by 18-June, earlier than in 2008. Flowers that were exposed to possible pollination until 25-June and then covered with a bag to prevent possible pollination from that date on produced 73 nuts indicating that pollination had substantially increased during the 7 day period after 18-June and before 25-June (Table 11 and Figure 7). This is the largest number of nuts observed so early in my studies. Flowers that were exposed to possible pollination until 2-July and then covered with a bag to prevent possible pollination from that date on produced 143 nuts again indicating that pollination had substantially increased during the 7 day period after 18-June and before 25-June and perhaps even a doubling of nut production between 25-June and 2-July (Table 11 and Figure 7). This was the largest nut set observed during my studies and demonstrates that production varies from year to year and that late-June and early-July are important times for pollination in the mid-Michigan region (Figure 7).

My results indicated that pollination was variable each year (2008-2010), that the initiation of pollination started at different times each year, and that there is a approximately 2-week period where successful pollination occurs and that pollen should be available during this time period for successful nut set (Figure 7). Pollen availability at other times is superfluous. However, the difficult part is determining when that two week time period will occur each year. In my three year study taken as a whole, the largest number of overlapping dates for this pollination period was days 176 to 192 (25-June through 10-July), indicating that pollination is

probably occurring each year between the last week of June and the first week of July in mid-Michigan.

Natural Pollination—removal of bags from flowers at various dates

To determine the end date of pollination, flowers were covered prior to pollen dehiscence. Using preset dates, bags were removed allowing exposure of flowers to pollen. Using this systematic approach I was able to determine the approximate end date of pollination.

In 2009, flowers that were covered preventing pollination until 26-June, 30-June, 6- July, 3-August, and 28-August produced 23, 19, 2, 3, 0 nuts, respectively. These data suggest that pollination was waning after 6-July and was terminated on or after 28-August (Table 12).

When combined with the 2009 data set from the "placement of bags on flowers at various dates" experiment, above (Table 11 and Figure 7), these data, suggest that pollination onset for 2009 was prior to 25-June and was essentially over by 6-July and completely finished by the end of August (Figure 8). It is not known if this reduction in pollination is due to a reduction of pollen or maturation of the female flowers.

Hand Pollination

To better understand the phenology of 'Colossal' flower receptivity and its effect on nut production, pollen was applied by hand to 'Colossal' flowers during pre-anthesis, anthesis, and post-anthesis. Therefore, 'Colossal' flowers received pollen one, two, or three time(s), which corresponded to these flower developmental stages.

In 2009, the number of nuts produced from hand-pollinated flowers ranged from 0 to 111 depending on the flower development stage(s) when pollinations were performed (Table 13). The highest number of nuts harvested (111) was produced from flowers hand pollinated at all

Table 12. Effect of pollination time on the total of nuts collected from European x Japanese chestnut trees cv. 'Colossal' growing in Eaton Rapids (Eaton County), Michigan (2009).

Treatment— Bag placed on flower [a]	**Total number of nuts**	**Treatment— Bag removed from flower** [b]	**Total number of nuts**
(date) 2009		**(date) 2009**	
25-Jun	8	**26-Jun**	23
29-Jun	50	**30-Jun**	19
5-Jul	64	**6-Jul**	2
2-Aug	45	**3-Aug**	3
__ [c]	—	**28-Aug**	0

a Dates on which 100 'Colossal' female flowers previously covered with pollination bags (Lawson #451) on 20-June were removed during the 2009 season. Pollination bag placed on 'Colossal' female flowers prevent pollination from the date of its placement over the flowers. Burs were harvested on 9-October during the 2009 season.

b Pollination bag removed from 'Colossal' female flowers allows pollination to takes place from the date the bag was removed.

c The sign (—) represents treatment not conducted.

.

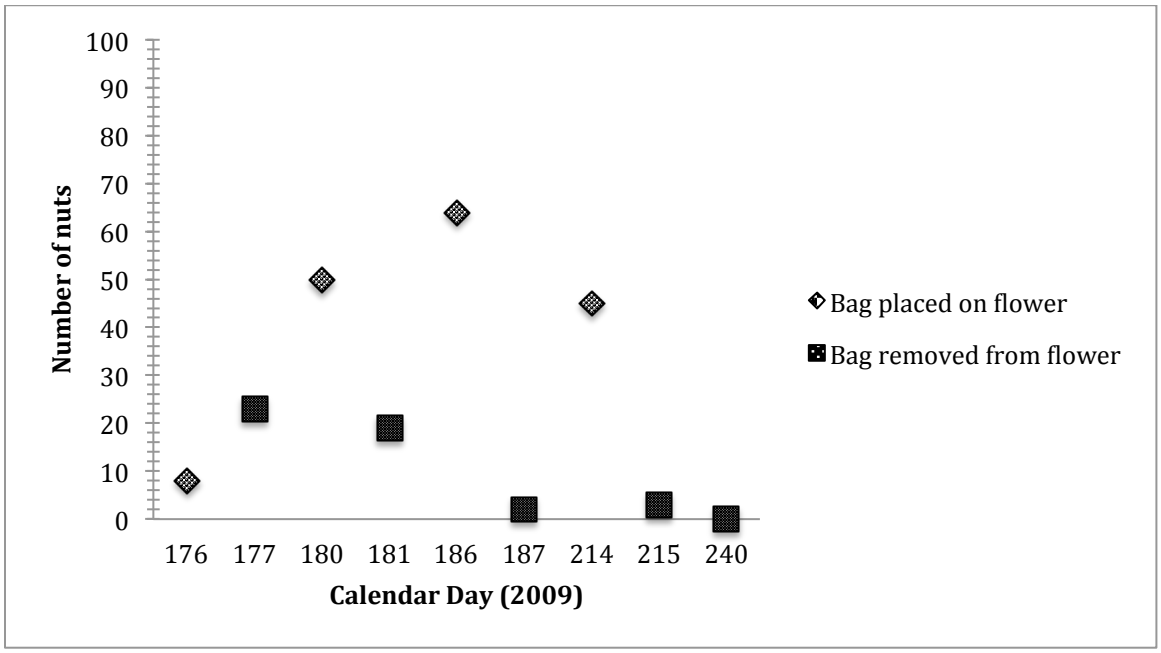


Figure 8. The number of nuts produced by flowers of European x Japanese chestnut cv. 'Colossal' when pollination was allowed to proceed by removing bags or blocked by adding bags at preset times. The number of nuts produced at each date with the procedure occurring at that date provided a pollination timeline for the 2009 season. The study was conducted once in 2009 through the duration of one blooming season. In 2009, nuts were first recorded on day 176 (25-June) and pollination was still occurring after 6-July but at a reduced rate. All pollination was over by 28-August. Nuts were harvested from trees growing in a single plot located in Eaton Rapids, MI. Calendar day represents the day of the calendar year the removal or addition of bags was performed.

flower development stages (+, +, +) (26-June, 3-July and 9-July). The number of nuts harvested from flowers receiving pollen at all three flower development stages was much greater than the number of nuts harvested from flowers pollinated once at pre-anthesis (60 nuts; +, –, –)(26-June); at anthesis (67 nuts; –, +, –) (3-July); and post-anthesis (33 nuts; –, –, +) (9-July). and somewhat higher than flowers pollinated twice at pre-anthesis and again at anthesis (74; +,+, –) (26-June and 3-July), at anthesis and again at post-anthesis (83; –,+,+) (3-July and 9-July), and at pre-anthesis and again post-anthesis (73 nuts; +, –, +) (26-June and 9-July). Flowers not pollinated (-, -, -) did not produce nuts. Statistically, there was no difference among these dates however there was a trend that a greater number of nuts were obtained when pollination was applied at anthesis. This experiment was repeated in 2010.

In 2010, the number of nuts produced from hand-pollinated flowers ranged from 4 to 86, depending on the flower development stage(s) when pollinations were performed (Table 13). The highest number of nuts harvested in 2010 was determined to have a different pollination pattern than in 2009. The highest number of nuts occurred when flowers were hand pollinated at anthesis and again at post-anthesis flower development stages (86; –, +, +) (24-June and 6-July). The number of nuts harvested from flowers receiving pollen at anthesis and post-anthesis development stages was greater than the number of nuts harvested from flowers pollinated once at pre-anthesis (32 nuts; +, –, –)(17-June); at anthesis (43 nuts; –, +, –) (24-June); and post-anthesis (17 nuts; –, –, +) (6-July), and only marginally higher than when flowers were hand pollinated twice at pre-anthesis and again at anthesis flower development stages (78; +, +, –) (17-June and 24-June). Those flowers hand pollinated at all flower development stages produced fewer nuts (63; +, +, +) (17-June, 24-June, 6-July) in comparison to the double pollinations. However, all four of the highest yielding pollination events included the flower development

Table 13. Effect of pollen application on the total number of nuts collected from European x Japanese chestnut trees cv. 'Colossal'. Pollen application was conducted one, two, three or four times. The + and – signs represent pollen application and no pollen application, respectively.

Year [a]	early pre-anthesis [b]	middle anthesis	late post-anthesis	very late late post-anthesis	Number of nuts collected [c]
2009	26-June	3-July	9-July	4-August	
	–	–	–	nc [d]	0
	+	–	–	nc	60
	–	+	–	nc	67
	–	–	+	nc	33
	+	+	–	nc	74
	–	+	+	nc	83
	+	–	+	nc	73
	+	+	+	nc	111
2009	23-June	2-July	7-July	4-August	
	–	–	–	–	0
	+	–	–	–	8
	–	+	–	–	23
	–	–	+	–	15
	–	–	–	+	0
	+	+	–	–	26
	+	–	+	–	17
	–	+	+	–	22
	+	–	–	+	9
	–	+	–	+	24
	–	–	+	+	9
	+	–	+	+	6
	+	+	–	+	26
	–	+	+	+	17
	+	+	+	–	41

a Pollen applications were conducted on the indicated dates per year. The experiment was conducted at two chestnut plantings located in East Lansing (Ingham County), and Clarksville (Ionia County), MI. The experiment was conducted two consecutives years: 2009 (East Lansing and Clarksville) and 2010 (East Lansing).

b Development stages of European x Japanese cv 'Colossal' flowers when pollen application was performed. All development stages were monitored each year to determine the date of pollen application.

c Number would indicate the number of nuts collected at the date the pollen was added.

d nc= not conducted.

Table 13. (cont'd)

Year [a]	early pre-anthesis [b]	middle anthesis	late post-anthesis	very late late post-anthesis	Number of nuts collected [c]
2009	23-June	2-July	7-July	4-August	
	+	+	+	–	41
	+	+	+	+	32
2010	17-June	24-June	2-July	4-August	
	–	–	–	nc	4
	+	–	–	nc	32
	–	+	–	nc	43
	–	–	+	nc	17
	+	+	–	nc	78
	–	+	+	nc	86
	+	–	+	nc	31
	+	+	+	nc	63

stage of anthesis similar to 2009. Those flowers hand pollinated at a time that did not include anthesis (pollinated at pre-anthesis and post-anthesis) produced the least number of nuts (31; +, –, +) (17-June and 6-July). As in 2009, statistically, there was no difference among these dates however there was a trend that the greater number of nuts were obtained when pollination was applied at anthesis.

Because nuts were obtained when flowers were hand pollinated at post-anthesis development stage, I wanted to determine if and when the flowers were no longer receptive. Using the same approach but adding a fourth developmental stage, hand pollinations were performed including a late post-anthesis development stage. This late post-anthesis development stage was 27 days after the post-anthesis development stage used in the other experiments (Table 13).

Flowers that were only pollinated at the late post-anthesis development stage produced no nuts indicating that flowers were no longer receptive 4 weeks after post-anthesis. In all other ways, the data were similar to the 2009 data set. The largest number of nuts was recorded when pollen was applied at all three (+, +, +, –) or all four development stages (+, +, +, +). Once again, the most important development stage for nut production is anthesis, the primary time for pollen receptivity. Only one out of 8 times when pollen was applied at anthesis was a lower yield recorded when compared to all other application development stages (Table 13).

In conclusion, it appears as if the highest number of nuts develop from flowers pollinated on late June and beginning of July. Thus, for an increase in the number of 'Colossal' nuts produced, pollinizer trees should release pollen from mid-June to mid-July.

DISCUSSION

Poor yield in fruiting trees have been associated with poor pollination. Factors affecting pollination can be grouped into weather conditions, flower composition, pollen quantity, pollen source, and genetic factors. Periods of steady rain, high humidity, and low wind currents during the blooming period of inflorescences hinder pollen transfer, especially among anemophilus trees, like chestnuts. The end result of poor pollination is a high number of empty cupule or burs, which reduces the productivity of chestnut orchards. Shi and Xia (2010) summarized that the main causes of low yield in Chinese chestnuts to be the high number of empty burs and the collection of less than three nuts per bur at harvest (when three nuts is the expected number of nuts produced by *C. mollissima* trees). However, they also stated that it is possible to find empty burs when sufficient amounts of pollen are available. Therefore, it may be difficult to determine the role of pollen and flower receptivity in season's with low yields but the better the process of pollination is understood, the better will be our understanding of an important aspect of nut production.

High chestnut yields in Michigan orchards may be affected by a combination of factors altering pollination rather than factors involving fertilization, embryo development and nut growth. To improve our understanding of chestnut reproductive phenology, pollination, and there relationship to nut production, I experimentally manipulated pollination and recorded nut production. For this study, nut production was considered the outcome of effective pollination events.

The period of 'Colossal' flower receptivity varied during the three growing seasons (2008, 2009, and 2010). The timing of pollen landing on female stigmatic surfaces was controlled by the use of pollination bags. Flowers blocked from receiving pollen between mid-June and early

July did not produce nuts and likewise flowers receiving pollen in late June and early July produced the largest yields. In some years, as observed in 2010 growing season, it appeared that pollen and receptive flowers were active as early as mid June, however, there were some exceptions. For example, in 2008, flowers covered on 26-June (late-June) did not produce any nuts, but taken in context, the 2008 season was problematic as yields were extremely low. Because in 2009 the first number of flowers were covered on 26-June, I cannot determine if 'Colossal' flowers were receptive to pollen as early as mid-June, as in the 2010 season. Possible explanations for this discrepancy are (1) adverse climatic conditions (*e.g*. consecutives days of cold and rain) could have interfered with pollen transfer and pollen landing on flower stigmatic surfaces, (2) the duration, the quantity and/or the quality of pollen grains released by the single pollinizer tree in the orchard during the days preceding the treatment (*i.e*. bagging flowers) were inadequate to efficiently pollinate the remaining receptive 'Colossal' flowers, and (3) the duration of the stigmata receptivity period was shortened in response to adverse climatic conditions experienced in 2008. During the 2002 growing season, Fulbright and Mandujano (*personal communication*) conducted a preliminary study in a naturally pollinated orchard to understand the relationship between duration of stigma exposure to the atmosphere (pollen), nut set and productivity. They observed a trend suggesting that the longer the stigma is exposed to pollen or the later a pollination bag was placed on flowers, the greater the chance of increasing the number of nuts per bur. Furthermore, results from the natural pollination experiment support the findings of Klinac et al (1995) in that nut production increases with the length of time stigmata surfaces were exposed to pollen. Klinac et al (1995) determined that the duration of the exposure time of chestnut flowers to pollen affects nut productivity. My results strongly suggest that it is not the duration of exposure but the greater chance of pollen arriving at anthesis. All of my data suggest

that anthesis is the critical time for pollination and therefore a large nut set. All of my data, including blocking pollen from flowers to exposing pollen to flowers suggest that there is a critical time for flower exposure to pollen. That time when studied by flower development stage suggests that anthesis is that critical time when pollen must be present. Miss that time and it does not matter how long the flowers are exposed to pollen, nut set will be reduced. Exposing flowers to pollen at anthesis ensures a good nut set and extending the exposure time will enhance nut set as long as anthesis is included in that time frame.

In summary, the optimum pollination time for 'Colossal' growing in Michigan orchards is when flowers are at anthesis, which can vary from year to year depending on the climatic conditions. From natural and pollination experiments, I established that the optimum pollination time for 'Colossal' flowers by 'Okei' pollen could be as early as 26-June and as late as 18-July. From hand pollination experiments performed at 3 or 4 flower development stages, I established that flowers pollinated more than once between 26-June and 9-July will result in high nut production as long as anthesis is included within that time frame. Together, these experiments have improved our understanding of 'Colossal' chestnut phenology and the role of monitoring flower development stage in relation to microclimatic conditions that Michigan orchards experience from year to year.

LITERATURE CITED

LITERATURE CITED

Akerman, J D. 2000. Abiotic pollen and pollination: ecological, functional, and evolutionary perspectives, in: Pollen and Pollination, Dafni, A., House, M., and E, Pacini (eds.). Springer Wien, New York.

Bai, Z. 1988. Report on the empty-cupule formation and its reducing experiment in the Chinese chestnut. Information of Forestry Science and Technology 7:5–9.

Costa, R., Ribeiro C., Valdivieso, T., Alfonso, S., Borges, O., Soerio de Carvalho, J., Costa, H A., Assuncao, A., Fonseca, L., Augusta C., Cruz, M H., Salazar, M., Matos Soares, F., Sequeira, J., Correira, P., and M J, Lima. 2008. Variedades de Castanha das Regioes Centro e Norte de Portugal-Projecto Agro 448. Instituto Nacional dos Recursos Biologicos I.P. INRB. I.P. (eds.) Oreiras, Portugal.

Dinis, L T., Ramos, S., Gomes-Laranjo, J., Peixoto, F., Vallania, R., Costa, R., and R, Botta. 2010. Phenology and reproductive biology in cultivar 'Judia' (*Castanea sativa* Mill). Acta Hort. 866:169–174.

Fulbright, D W., and M, Mandujano. 2001. Pollination of New Michigan Chestnut Orchards in 2000 and 2001, in: 92nd Annual Report of the Northern Nut Growers Association, Northern Nut Growers Asson.,Inc. Cornell, New York.

Fulbright, D W., Mandunajo, M., and S, Stadt. 2010. Chestnut production in Michigan. Acta Hort. 866:531–537.

Hunt, K L., Gold, M A., and M R, Warmund. 2005. Chinese Chestnut Cultivar Performance in Missouri. Acta Hort. 693: 145–148.

Klinac, D J., Lelieveld, F., and R D, Knowles. 1995. Time of flowering and pollination in New Zealand chestnut selections, and introduced cultivars. New Zealand J. Crop Hort. Sci. 23:331–340.

Mert, C., and A, Soylu. 2006. Flower and stamen structures of male-fertile and male-sterile chestnut (*Castanea sativa* Mill.) cultivars. J. Amer. Hort. Sci. 131:752–759.

Miller, G. 2003. Chestnuts, in: A guide to nut tree culture in North America. Fulbright, D. (ed.). Volume 1. Northern Nut Growers of Asson., Inc. Cornell, New York.

Nakamura, M. 1992. Elongation of styles and optimum pollination tin in Japanese chestnut (*Crenata sativa* Sieb. & Zucc.). (Summary in English) J. Japan. Soc. Hort. Sci. 61:265–271.

Nakamura, M. 2001. Pollen tube growth and fertilization in Japanese chestnut (*Castanea crenata* Sieb. & Zucc.) J. Japan. Soc. Hort. Sci. 70:561–566.

Nyeki, J. 1996. Pollination and Fertilization, in: Floral biology of temperate zone fruit trees and small fruits. Nyeki, J., and M, Soltesz (eds.). Akademiai Kiado, Budapest.

Rutter, P A. 1996. Chestnut pollinator's guide. Bulletin 1, Badgersett Research Corporation, Pensylvania.

Shi, Z., He, C., and H, Tang. 2003. Study of the precocity of the Chinese chestnut in Yunnan. Nonwood Forestry Res. 21:11–16.

Shi, Z., and R, Stosser. 2005. Reproductive biology of Chinese chestnut (*Castanea mollissima* Blume). Europ. J. Hort. Sci. 70:96–103.

Shi, Z., and L, Xia. 2010. Stigmatic morphology of Chinese chestnut (*Castanea mollissima* Blume). HortSci. 45:981–983.

Soltesz, M. 1996. Flowering, in: Floral biology of temperate zone fruit trees and small fruits. Nyeki, J., and M, Soltesz (eds.). Akademiai Kiado, Budapest.

Wang, G P., Yang, Q., Zhang, K., and W M, Ciesla. 2001. Factors affecting production of Chinese chestnut in Xinxian County, Henan Province, China. Forestry Chronicle 77: 839–845.