ABSTRACT

BOWLES, MOLLIE ELLEN. Interactions Between *Phytophthora* spp. and *Castanea* spp. and the Creation of a Genetic Linkage Map for the F1 Parent in a First-Generation Backcross Family of *Castanea* spp. (Under the direction of John Frampton.)

Efforts are underway to restore the American chestnut, *Castanea dentata* (Marsh.) Borkh, to its original range within the United States. The American Chestnut Foundation has traditionally focused on introducing blight resistance of the Chinese chestnut, *C. mollissima* Blume, into their breeding stock. Now, however, they are encountering problems wit h a root rot caused by *Phytophthora cinnamomi* Rands, and the threat of a potential disease caused by *Phytophthora ramorum* Werres, De Cock, and Man in't Venld. In order for the Foundation to breed for resistance against the effects of these pathogens, much more needs to be discovered regarding their interactions within *Castanea*. This thesis is part of the effort to understand these interactions. The first chapter provides a literature review of interactions for two *Phytophthora* species and *Castanea* spp. The second chapter presents experiments investigating the mode of inheritance and number of loci controlling resistance to each of these *Phytophthora* species. Initial experimentation appears to suggest that resistance to *P. cinnamomi* may be controlled by a single dominant gene in chestnut, but conclusive evidence from subsequent experiments was not found; possible causes are discussed. A series of screening assays based on leaf disk assays using *P. ramorum* also failed to provide conclusive evidence that a single locus controls the degree of response, but statistically significant differences were noted among seedlots in each trial, suggesting some level of genetic control. The third chapter presents a genetic linkage map developed from amplified fragment length polymorphic (AFLP) markers for the F1 parent in a backcross family ((*C. mollissima* 'Mahogany' x *C. dentata* 'RCF1' = 'KY115') x *C. dentata* 'WB348'). This purpose of this map is to correlate future resistance segregation patterns from controlled *P. cinnamomi* inoculation trials with specific marker loci. This would provide a foundation for future genetic research regarding the number of loci controlling resistance to root rot caused by *P. cinnamomi* and its relationship with other traits of interest in chestnuts.

INTERACTIONS BETWEEN *PHYTOPHTHORA* spp. AND *CASTANEA* spp. AND THE CREATION OF A GENETIC LINKAGE MAP FOR THE F1 PARENT IN A FIRST-GENERATION BACKCROSS FAMILY OF *CASTANEA* spp.

By

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BIOGRAPHY

Mollie Ellen Bowles was born on December 20, 1980, in Concord, North Carolina. In 2003, she graduated *summa cum laude* from Samford University in Birmingham, Alabama, with a BS in Environmental Science, a concentration in Geographical Information Systems, and a minor in business. As an undergraduate, her work mainly focused on stream ecology, management of tropical and native plants, and surveys for local endangered animal species. During an internship with the Cawaco Research Conservation and Development Council, she authored a watershed restoration plan for the Five Mile Creek watershed, which covers 78 square miles in Jefferson County, Alabama; recommendations from this plan are still being followed. Mollie entered North Carolina State University in the fall of 2003 to obtain her Master of Science degree in Forestry. For a time during the pursuit of this degree, she contemplated switching careers to travel throughout the States with friends playing and selling musical instruments. However, Mollie did finally finish her research project. After her defense, she will be moving to the mountains of North Carolina to marry Dr. Anthony Vincent LeBude in August 2006. Mollie looks forward to life in the mountains and to finding work that will enhance the knowledge, skills, and abilities that she has developed during her educational degrees.

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CHAPTER 1
Introduction to Diseases of Chestnuts (*Castanea* spp.) Caused by *Phytophthora cinnamomi* and *Phytophthora ramorum*

Progress is underway to restore American chestnut, *Castanea dentata* (Marsh.) Borkh, to its original range within the United States. Current efforts by The American Chestnut Foundation are focused on establishing resistance to the chestnut blight, caused by *Cryphonectria parasitica*, (Murrill) Barr, within its breeding stock (Ellingboe, 1994, Sisco 2004). Recently, the Foundation has become concerned about two species of *Phytophthora* that threaten their success. *Phytophthora cinnamomi* Rands, was introduced into the United States in the 1700s and has slowly become established throughout the southern range of American chestnut. This pathogen is a serious threat, as it is known to kill entire stands of infected American chestnut (Butterick 1913). The second pathogen which may threaten the success of restoration efforts is *Phytophthora ramorum*, Werres, De Cock, and Man in't Venld. This species has rapidly become established on both the European and North American continents since 1995 and has been more recently introduced into the eastern United States (Rizzo *et al*. 2002.). While relatively little is known about its effect on American chestnut, its impact on other species, especially those of the oak family, has generated serious concern regarding its potential effect on American chestnut.

While post-introduction management of both species of *Phytophthora* is potentially possible through chemical and cultural practices, such efforts are costly, labor-intensive, and only temporarily effective. A more effective approach would be to develop resistant stock, using an interspecific backcross breeding strategy to capture the genetic resistance from closely related species (Burnham *et al.* 1986). Because of its resistance to *C. parasitica,* Chinese chestnut is already being used by the Foundation to establish blight resistance in American chestnuts (Ellingboe 1994; Hebard 1994; Sisco 2004). Because this breeding program is already in place, and because Chinese chestnut is also resistant to *P. cinnamomi* (Crandall *et al.* 1945), it is practical to search existing lines of breeding stock for resistance to root rot caused by *P. cinnamomi*. It would be a bonus if Chinese chestnut were not reactive to *P. ramorum*, as breeding could be selective for reduced reaction levels to this pathogen as well.

In order to employ the backcross breeding method most effectively, much more needs to be discovered regarding the interactions of these *Phytophthora* species with the Chinese and American chestnut species. Each chapter in this thesis is part of a series of efforts to understand these interactions. The first chapter provides a literature review covering historical interactions between the species, and genetic research already performed within the genus *Castanea*. The objective of the second chapter is to investigate the mode of inheritance and number of loci controlling resistance to both *Phytophthora* species. To this end, a series of controlled inoculation experiments are presented which employ several breeding lines held by The American Chestnut Foundation, and the resistance contained within each of these lines is discussed. The objective of the third chapter is to present a genetic linkage map created from amplified fragment length polymorphic (AFLP) markers for the F1 parent ('KY115') in a first generation backcross family in the *C. mollissima* 'Mahogany' line. This purpose of this map is to correlate specific marker loci with disease phenotype (resistant/not resistant) disease segregation patterns to be generated in future controlled-inoculation trials.

1.1 *Phytophthora cinnamomi*

~~1.1~~1.1.1. Historical Perspective

Phytophthora cinnamomi is thought to have originated in central and southern regions of the South Pacific (Day 1932, McRae 1932 *rptd. in* Crandall *et al.* 1945). It is believed to have been introduced into the United States through a southern port in the mid-1700's (Crandall and Gravatt 1967 *rptd. in* Zentmeyer 1980, Zentmeyer 1977). Since its introduction, *P. cinnamomi* has spread across most of the Coastal and Piedmont regions of the eastern states from Maryland to Mississippi, as well as some lower-altitude regions of the Appalachians (Gravatt and Crandall 1945; Tainter and Baker 1996; Benson and Grand 2000).

Some of the earliest reports of the death of native chestnut stands due to *Phytophthora*-like symptoms are *ca*. 1825, and a marked decline in the overall chestnut population was reported around 1840 (Clinton 1912; Butterick 1913; Crandall *et al.* 1945). It was not until 1930, however, that *P. cinnamomi* was first

identified in the United States (White 1930). A year later, this pathogen was associated with the "ink disease" of *C. dentata* (Gravatt and Crandall 1945).

Butterick's manuscript (1913) is likely the earliest and most complete account of the "chestnut decline" at the stand and regional levels. He noted that in about 1840, chestnuts had begun dying in eastern North Carolina and were still disappearing west of the Blue Ridge in 1913. Although several causes were initially attributed to the decline of chestnuts throughout the South, including insects, fire, and silvicultural and agricultural practices (Butterick 1913; Mason 1912), the phenomenon of the chestnut decline proper, was easily divisible into three stages not indicative of these causes (Butterick 1913). During the first stage, which lasted 5 to 10 years, about 80% of chestnuts within a 12.95 km^2 (5 mi^2) stand would die in an exponential fashion. Most trees died within a single season after infection, though some struggled for two. At the start of the second stage, only the smallest sprouts and seedlings would remain, growing vigorously until they reached approximately 10-15 cm (4 – 6 in) in diameter, at which point most would become infected and die within the next season. The third stage of regional decline encompassed the death of outlier trees, such as pasture and dooryard trees and any remaining progeny of the original stand.

Since 1912, almost all of the remaining specimens of *C. dentata* in North Carolina have been located in isolation and are principally found on mountain ridges and higher elevations where *P. cinnamomi* has presumably not been introduced (Mason 1912; Butterick 1913; Sisco 2004). More recently, the percentage of Fraser fir Christmas trees infected with *P. cinnamomi* in plantations in North Carolina's mountain counties has been reported at about 9%, with a range of 0-90% (Grand and Lapp 1974; Benson and Grand 2000).

1.1.2. Disease Biology

Phytophthora cinnamomi is classified as a water mold. Taxonomically, it is placed in the family Pythiaceae, order Pythiales, class Oomycetes of the phylum Protista. Mycelium and zoospores of *P. cinnamomi* spreads short distances through the soil. Mycelia can also spread inter- and intra-cellularly in

infected plant host tissue (Reeves 1974). Mycelial growth and disease occurrence can be inhibited by the interaction of certain soil microorganisms with *P. cinnamomi*, including three species of *Streptomyces,* five species of basidiomycetes, and several other miscellaneous species (Finlay and McCracken 1991; Branzanti *et al.*1999).

Sporangia of *P. cinnamomi* form in non-sterile media, and may germinate directly *via* a germ tube or, at cooler temperatures, indirectly through the production of up to 30 motile zoospores (Zentmeyer 1980). Zoospores are attracted to root tips through electrotaxis and chemotaxis (Zentmeyer 1980; Gow 2004). Chlamydospores form within roots or soil as survival structures during unfavorable conditions; these thick-walled sections of hyphae may produce up to 10 germ tubes, which become mycelia (Zentmeyer 1980).

Sexual reproduction of *P. cinnamomi* is heterothallic, typically requiring the presence of two mating types, A1 and A2, for production of oospores (Zentmeyer 1980). However, two exceptions to this general rule have been reported. First, mitotic reduction and the development of mature oospores may occur under certain circumstances, for example, in old cultures, or young cultures exposed to very moist soil conditions; other circumstances include chemical stimulation by root exudates and mechanical stimulation of the A2 type compatibility group by artificial damage or by damage caused by parasites such as *Tricoderma viridae* Pers. ex Fries (Reeves and Jackson 1972 *rptd. in* Reeves 1974; Zentmeyer 1980). Second, interspecies hybridization may occur when hyphae of *P. cinnamomi* meet opposite mating types of *P. cryptogea* Pethybr. and Laff., *P richardiae* Buism., or *P. palmivora* Butler meet (Zentmeyer 1980).

P. cinnamomi can function as a saprophyte or a parasitic plant pathogen (Zentmeyer 1980; Sinclair and Lyon 2005). In the latter mode, it penetrates epidermal cells directly or invades through host wounds (Gow *et al.* 1999). In chestnuts and other hosts, this pathogen feeds on the inner root cortex primarily of feeder roots, secondarily in support roots, and may also progress a short distance up the trunk in the cambial layer (Zentmeyer 1980; Day 1938a). Evidence suggests that different isolates may vary in aggressiveness (Zentmeyer 1980; Benson and Grand 2000).

The means of dissemination for *P. cinnamomi* are through soil water and anthropogenic transport. Shallow, infertile, degraded, eroded, poorly drained, or poorly managed soils are described as the most likely sites for disease development (Campbell and Copeland 1954). Site-specific disease patterns generally trace soil moisture drainage patterns (Zentmeyer 1980). The pathogen is readily transported across long distances *via* rootstock or through mechanical means such as unwashed equipment (Sidebottom 1998). The sheer number of host species and the pathogen's ability to live as a saprophyte contribute greatly to its widespread survival and persistence (Erwin *et al.* 1983).

In general, host symptoms of the infection by *P. cinnamomi* include feeder root decay, root and collar rot, shoot desiccation, and whole-plant mortality (Zentmeyer 1980). Additional symptoms specific to chestnuts include a characteristic sunken black ring girdling the collar of the plant, and an inky black substance that may be exuded at the base, giving the disease its nickname "ink disease" (Sisco 2004). In chestnuts, decline and death usually occur within a single season, often in as little as a few weeks, though trees may decline over a period of several years (Butterick 1913). It has been shown, however, that certain species of ectomycorrhizal fungi (*Laccaria laccata* (Scop.) Cooke, *Hebeloma crustuliniforme* (Bulliard) Quélet, *H. sinapizans* (Bulliard) Quélet, and *Paxillus involutus* (Fries) Fries) have the ability to reduce the negative effect of *P. cinnamomi* in the *Phytophthora*-susceptible European chestnut, *C. sativa* (Branzanti *et al.* 1999).

1.1.3. Options for Control

Control of the affects of *P. cinnamomi* through cultural or chemical practices such as trunk injections, soil fumigation, or aerial spraying has been limited (Gravatt and Crandall 1945; Marchetti and D'Aulerio 1998; Colquhoun *et al.* 2000; Benson and Grand 2000; Tynan *et al.* 2001). Such measures only provide limited protection for up to two years for orchard crops, four years for eucalypts in the field, and 4-6 months for Fraser fir in nursery transplant beds (Shears and Fairman 1997 *rptd. in* Department of Conservation and Land Management 1999; Benson and Grand 2000). Another drawback to such measures is leaf burn in

sensitive neighboring plants when chemicals are applied on a large scale (Department of Conservation and Land Management, Australia 1999). Additional environmental, financial, logistical, and public relations concerns (Colquouhn *et al.* 2000) indicate that it would be impractical to assume that such culturally intensive control measures could be applied on a broad scale throughout the Eastern United States. A better approach to ensure the success of reintroducing *C. dentata* throughout its original range would be to develop resistant trees through selective breeding lines.

1.1.4. Models of Resistance Within the Pathosystem

Early research (Milburn and Gravatt 1932; Crandall *et al.* 1945; Gravatt and Crandall 1945) investigated the pathogenicity of *P. cinnamomi* on twelve of the thirteen species of chestnut and chinquapin. Highly susceptible species included American chestnut, *C. dentata*, European chestnut, *C. sativa* Mill., and six species of chinquapin native to North America. Highly resistant species included the four Asiatic species: the Japanese chestnut, *C. crenata* Sieb. and Zucc., the Chinese chestnut, *C. mollissima* Blume, the Senguin chestnut, *C. senguinii* Dode, and the Henry chinquapin, *C. henryi* (Skan.) Rehd and Wils. (Table 1.1) The results of trials using excised tissues are somewhat less reliable (Day 1938b; Borrod 1971; Salesses *et al* 1993; Robin and Desprez-Loustau 1998) but virtually all field experience confirms that *C. dentata* is highly susceptible to *P. cinnamomi* root rot (Sisco, P.H. personal communication, Hebard, F.V., personal communication).

Within a given pathosystem, heritable disease resistance can be classified as either vertical or horizontal (Vanderplank 1984). Vertical resistance involves the interaction of one or a few host resistance genes with specific corresponding pathogen genes (virulence/avirulence genes) and plants possessing vertical resistance may be nearly immune to a particular disease (Vanderplank 1984; Erwin and Ribeiro 1996). Horizontal resistance is polygenic in nature; that is, it is controlled by multiple small effects host resistance genes (Nelson 1980; Erwin and Ribeiro 1996). Horizontal resistance provides rate limiting resistance, rather than true immunity (Erwin and Ribeiro 1996). Host horizontal resistance is expressed with all isolates of a pathogen, while expression of vertical resistance is isolate dependent.

Erwin and Ribeiro use the terms horizontal and general resistance, and the terms specific and vertical resistance interchangeably (1996). According to their reviews, most instances of host resistance against *Phytophthora* spp. are horizontal, with relatively few cases exhibiting vertical resistance. Examples of pathosystems displaying horizontal resistance to root rot caused by *P. cinnamomi* include acacia, avocado, *Banksia* spp., *Eucalyptus* spp., *Pinus* spp., and *Rhododendron* spp. Examples of vertical resistance include interactions between strawberries and *P. fragariae* Hickman, potatoes and *P. infestans* (Mont.) de Bary, tobacco and *P. parasitica* var. *nicotianae* Datsur, soybean and *P. sojae* Kaufmann and Gerdemann, apple and *P. syringae* (Klebahn) Klebahn, and Adzuki beans and cowpeas and *P. vignae* Purss (Erwin and Ribeiro 1996).

1.2 Phytophthora ramorum

1.2.1. Historical Perspective

By the time *Phytophthora ramorum* and its high aggressiveness were formally recognized, it had already become established in European nurseries and in wildlands on the Pacific coast of the United States. Unusual symptoms of twig blight and mortality were first identified on rhododendron in Germany and the Netherlands in 1993 and the causal pathogen was named *Phytophthora ramorum* in 2001 (Werres *et al.* 2001). In 2002, *P. ramorum* was identified as the causal agent of an epidemic mortality in several oak species that had been in progress in California for seven years (Rizzo *et al.* 2002). Because this pathogen has a large number of hosts (APHIS 2006a), and because it may be disseminated aerially or through water or soil transport (Davidson *et al.* 2005), *P. ramorum* has the potential to decimate a relatively broad spectrum of host species. By 2005, isolations of *P. ramorum* from symptomatic plants were reported in Florida, Georgia, Louisiana, Tennessee, and South Carolina (Kent 2005). The potential for establishment in these and other states in the southeastern United States is considered high due to comparable ecological environments and frequent importation of horticultural plants from infected regions (Kent *et al.* 2004).

1.2.2. Disease Biology

Phytophthora ramorum is classified similar to *P. cinnamomi,* produces chlamydospores and oospores, and is a heterothallic species with A1 and A2 mating types. Although the A1 mating type is by far the dominant type found on the European continent, it has so far been limited to nursery stock within the United States, where only the A2 mating type has been isolated from the field (Garbelotto *et al*. 2003; Hansen *et al.* 2003; Werres and de Merlier 2003; Ivors *et al.* 2004; Werres and Kaminski 2005; Ivors 2006). It is important to emphasize, however, that the establishment of sexually reproducing populations in any location may lead to an increased genetic variation within the pathogen and the development of either increased aggressiveness, new host species, or both.

In general, growth of the A2 mating type is slower and more variable than the A1 type and tends to produce broader sporangia and more variable morphological features (Hüberli, personal communication *rptd. in* Hayden *et al.* 2004; Werres and Kaminski 2005). Contradictory reports exist regarding differences in the aggressiveness of each mating type. A2 isolates from the United States are reportedly less aggressive than A1 isolates on northern red oak (*Quercus rubra*) and rhododendron (Brasier 2003), yet minimal statistical differences were found regarding the mating type aggressiveness in another inoculation trials involving rhododendron (Tooley *et al.* 2004). Other studies suggest that slow-growing A2 isolates showed low virulence, while A1 and fast-growing A2 isolates showed greater pathogenicity on rhododendron, although these differences were questionable due to the possible effects of subculturing (Werres and Kaminski 2005).

Transportation of *P. ramorum* spores has been observed aerially *via* rain splash and perhaps through wind currents as well, as 5-15 m longitudinal transport and 25 m vertical transport have been reported; dissemination can also occur through streamwater (up 1 km transport), and the transportation of infested soil or plant material (Davidson *et al.* 2005; Rizzo *et al.* 2005; APHIS 2006a). Inoculum propagules are generally not produced on oak species, but on other forest species that suffer non-lethal infections and may serve as long-term reservoirs (Garbelotto *et al.* 2003; Davidson *et al.* 2005).

By 2003, 23 species in 12 plant families were found to be naturally infected with *P. ramorum*, and more were identified as potential host species in controlled inoculation trials (Davidson *et al*. 2003). Currently, 100 plant species in 55 genera are listed for quarantine regulation in the United States; species within the Ericeae and Fagaceae families are particularly prone to infection (APHIS 2006a). Examples of host genera include *Quercus*, *Lithocarpus, Acer*, *Lonicera*, *Rhododendron*, *Rosa*, *Sequoia*, *Pseudotsuga*, *Viburnum*, *Abies*, *Magnolia*, *Rubus*, and *Toxicodendron* (APHIS 2006a). It is reasonable to assume that many more species will be added to this list as the pathogen becomes more established and the full range of host species is discovered.

Oak species that have been placed on the federal quarantine list include tan oak, *Lithocarpus densiflora* (Hooker and Arnold) Rehder, canyon live oak *Quercus. chrysolepis* Liebm., southern red oak *Q. falcata* Michaux, Holm oak, *Q. ilex* L., coast live oak, *Quercus agrifolia* Née, California black oak, *Q. kelloggii* Newberry, Shreve's oak, *Q. parvula* Greene var. *shrevei* (C.H. Muller) Nixon, European turkey oak, *Q. cerris* L., sessile oak, *Q. petraea* (Mattuschka) Liebl., and northern red oak *Q. rubra* L. (APHIS 2006a). The disease complex associated with oaks appears to be limited to the red oak group (Erythrobalanus) and absent from the white oak group (Lepidobalanus) (McPherson *et al.* 2005). Other members within the oak family (Fagaceae) that are federally listed for quarantine regulation include European beech (*Fagus sylvatica* L.) and European chestnut (*Castanea sativa* Miller) (APHIS 2006a).

Artificial inoculation trials using both mating types in trials of European chestnut (Denman *et al.* 2005a) and the A2 mating type in trials of American chestnut (Hansen *et al.* 2005) indicate that at least some species within the genus *Castanea* are susceptible to *P. ramorum* in inoculation trials. Methods of trial inoculations with *P. ramorum* have included stem inoculations and wounded and non-wounded leaf dip studies. Lesion sizes resulting from stem inoculations of American chestnut were comparable to canyon live oak, California black oak, and red oak, but were only 40% the size of lesions measured for tan oak, which is highly susceptible (Hansen *et al.* 2005).

The wounded and non-wounded leaf trials of European chestnut were conducted by immersing distal and apical portions of detached leaves, respectively, into zoospore suspensions (Denman *et al.* 2005a). Seventy-five percent of the non-wounded chestnut leaves and 100% of the wounded chestnut leaves provided reisolation rates greater than 75% (Denman *et al.* 2005a). In comparison to other genera tested in that trial, European chestnut showed relatively little development of necrosis, but a higher reisolation rate (Denman *et al*. 2005a) As with other species, younger chestnut leaves were found to be much more susceptible to infection, while older leaves are generally less susceptible. These trends have been observed in both attached and detached artificial leaf inoculation trials and could suggest that chestnuts may serve as important inoculum reservoirs rather than mortally susceptible hosts (Denman *et al.* 2005a; Hansen *et al.* 2005).

1.2.3. Molecular Research

The identification of *P. ramorum* through restriction fragment length polymorphisms (RFLPs) and polymerase chain reaction (PCR) products has been well established (Garbelotto *et al.* 2002; Hayden *et al.* 2004; Kong *et al.* 2004; Martin and Tooley 2004; Martin *et al.* 2004; Tooley *et al.* 2005) and approved by the United States and The Netherlands as a diagnostic tool (Hayden 2004). For PCR methods, two rounds of PCR using genus-specific primers in the first round and species-specific primers in the second are used (Martin and Tooley 2004). This technique is sensitive enough to detect *P. ramorum* in the exudates of bleeding oaks, which is not possible using traditional culturing techniques (Hayden 2004).

Several molecular marker systems have been developed for use in determining mating types and phylogenetic relationships as well as for genomic mapping (Dodd *et al.* 2005; Prospero *et al.* 2004; Kroon *et al.* 2004; Ivors *et al.* 2004; Tyler 2005). Seven microsatellite loci have been detected which differentiate between mating types (Prospero *et al.* 2004). Other SSR loci allow the identification of five genotypes within the European continent, compared with only one genotype in the United States (Rizzo *et al.* 2005).

Single nucleotide polymorphisms (SNPs) have been used to denote differences between European and North American isolates, but not between mating types (Kroon *et al.* 2004). The use of simple sequence repeat (SSR) loci has allowed the identification of five genotypes within the European continent, compared with only one genotype in the United States (Rizzo *et al.* 2005). Amplified fragment length polymorphisms (AFLPs) have been used to indicate close relationships between *P. ramorum*, *P. lateralis* Tucker and Milbrath, and *P. hibernalis* Carne (Ivors *et al.* 2004).

A significant achievement has been made through the use of approximately 200,000 SNPs to map the genome of *P. ramorum* across 65 Mb at a depth of 7x, using a whole genome shotgun approach (Tyler 2005). The completion of this genome sequencing project for *P. ramorum* has laid the foundation for important advances in the genomic research in this species.

1.2.4. Options for Control

Various methods of control have been established for the treatment of infested plant material, soil, and other inanimate surfaces. Completely drying or composting small amounts of woody debris under commercial (heated) composting conditions for two weeks appears to kill *P. ramorum* cultures and spores (Garbelotto 2003), but these control methods are not federally approved (APHIS 2006b). Federally approved methods for soil sterilization include heating at 82.2°C (180°F) for 30 minutes, or treatment with chloropicrin, dazmet, metam-sodium, or methyl bromide. Procedures for treating infected plant material include incineration, burial at depths greater than 2 m (6 ft), and steam sterilization (APHIS 2006b). Federally approved procedures for treating run-off and irrigation water, nonporous and wood surfaces and for disinfecting tires, shoes, and other equipment also exist (APHIS 2006b).

Several compounds have been found which limit or prevent *P. ramorum* disease development. Grapefruit extract and, to a lesser extent, chitosan, have been found to limit colony growth and sporulation of zoospores of *P. ramorum* on rhododendron (Orlikowski 2003, 2004a). Chemical fungicides were found to have varying effects on the development and spread of ramorum twig blight on rhododendron. The most

effective compound found so far has been furalaxyl when applied 48 hours before or after inoculation; fosetyl (fenamidone+fosetyl-A1) was the next most effective, while oxadixyl+mancozeb and cymoxanil+famoxate were found to be somewhat less effective (Orlikowski 2004b). Metataxyl is particularly effective in controlling the size of *P. ramorum* colonies grown *in vitro*; other compounds, such as copper sulfate and phosphorous acid are less effective, but may prove useful *in vivo* (Garbelotto *et al.* 2002).

The application of chemical and cultural treatments (such as through foliar sprays or trunk injections) to control *P. ramorum* infections in non-nursery settings may be limited due to the lack of a time-efficient, long-lasting, and cost-effective treatment protocol, as well as a general lack of public support for aerial applications; in addition, the effects of a broad-scale treatment regime on other micro- and macro-organisms is unknown (Rizzo *et al.* 2005). Suggested control measures within forest settings include placing infected nurseries and forests under quarantine, eradicating infected host plants through, clearing potential host species through fire or harvesting, and using non-host or genetically resistant species for reforestation efforts (Rizzo *et al.* 2005).

1.2.5. Models of Resistance Within the Pathosystem

There are two types of host response to *P. ramorum*, which are dictated according to the host species. In virtually all susceptible species *except* oaks, host reactions appear limited to the dieback of infected leaves and branches, and the association is commonly referred to as ramorum twig blight. The more dramatic disease symptoms are commonly known as sudden oak death and involve the infection of oak species, particularly tanoak, but also coast live oak, California black oak, and Shreve's oak. The events associated with sudden oak death in California are as follows: rapid foliage color change; bleeding of a viscous red to black exudate from apparently intact bark close between the soil line and up to 2 m high; infestation and tunneling by Scolytidae beetles (either the western oak bark beetle, *Pseudopityophthorus pubipennis* LeConte, the oak ambrosia beetle, *Monarthrum scutellare* LeConte, or the minor oak ambrosia beetle, *M.*

dentiger LeConte); infection with *Hypoxylon thouarsianum* (Lév.) Lloyd in the bleeding areas; and plant death (McPherson *et al.* 2001; McPherson *et al*. 2005).

Since this pathogen does not cause wood decay, it has been suggested that the breakage of infested boles is caused by extensive tunneling by the beetles, as the beetles are known to bore up to 10 cm into the bole (in comparison, *P. ramorum* has not been reported at depths greater than 3 cm) (Rizzo *et al.* 2002; McPherson *et al.* 2005). In one study, all coast live oaks under investigation were infested with beetles before whole-plant mortality occurred (McPherson 2001). It has also been estimated that beetle infestations decrease the survival period for oak hosts from 7-9 years (when infested with *P. ramorum* alone) to less than 3 years (McPherson *et al.* 2001; McPherson *et al*. 2005).

The severity of the disease across populations of oak species had led to concern over extinction events, especially for tan oaks (Davidson *et al.* 2005; McPherson *et al.* 2005). For the somewhat less susceptible coast live oak, within-population mortality resistance has been noted to a much greater degree than between-population resistance, which may indicate control by several gene loci (Dodd *et al.* 2005). It has been suggested that the losses from this and other oak species which demonstrate within-population resistance (that is, oak species besides tanoaks) may stabilize over time due to natural selection pressures (McPherson *et al.* 2005).

Resistance is less well defined for tree species other than oaks, which exhibit non-lethal dieback infections. Reports of infection on mature specimen European chestnut have been described as non-lethal infections of leaves with soft thin-cuticles and that are located on understory epicormic shoots of mature trees (Denman et al. 2005b). Because the described incidences have been limited to foliar and leaf dieback and because the pathogen has been noted to sporulate prolifically on the leaf surfaces (Denman *et al.* 2005a, Denman *et al.* 2005b), it can be theorized that *P. ramorum* infection of European chestnut is a non-lethal infection type, but that susceptible chestnut species may serve as reservoirs for the pathogen. Similar roles have been observed in bay laurel and small tanoaks in the forests of the western United States (Garleotto *et al.*

2003; Maloney *et al.* 2005). Resistance in bay laurel to non-lethal ramorum dieback has been demonstrated both within and between populations (Rizzo *et al.* 2005), which may indicate the control of resistance reactions by multiple genes. More research is needed before the number of genes and mode of inheritance could be reliably described for resistance in the genus *Castanea*.

1.3 Prior Genetic research within *Castanea* spp.

A great deal of molecular research has been achieved in the chestnut genome. Efforts to efficiently type European chestnut cultivars has progressed through the analysis of protein and albumin polymorphisms (Alvarez *et al.* 2000; Alvarez *et al.* 2003; Martín *et al.* 2005a), allozymes and isozymes (Villani *et al* 1999a; Villani *et al.* 1999b; Pereira *et al.* 1999; Gualão *et al.* 2001; Aravanopoulos and Drouzas 2005), random amplification of polymorphic DNA (RAPDs) (Seabra *et al.* 1996; Oraguzie *et al.* 1998; Paffetti *et al.* 1999; Santana *et al.* 1999; Valdiviesso 2000; Goulão *et al.* 2001; Kubisiak and Roberds 2005), and simple sequence repeats (SSRs) (Botta *et al.* 1999; Buck *et al.* 2003; Marinoni *et al.* 2003; Yamamoto *et al.* 2003; Costa *et al.* 2005; Martín *et al.* 2005b; Beccaro *et al.* 2005; Bounous *et al.* 2005; Kubisiak and Roberds 2005). Molecular typing in interspecific *C. sativa* x *C. crenata* crosses has also been achieved (Santana *et al.* 1999). At least two research groups have investigated the similarity between chestnut species and other plant species from other genera (Connors *et al.* 2001; Barreneche *et al.* 2004). Long-term management plans for gene conservation are also being developed (Eriksson *et al.* 2005; Alexander *et al.* 2005; San-José *et al.* 2005; Jorquera *et al.* 2005).

Isozyme analysis and comparisons of genetic diversity have suggested that the Chinese chestnut is the progenitor of *Castanea* (Lang and Huang 1999). Further studies based on polymorphic RAPD data among cultivars suggest that there is a high degree of genetic variation at the species level (Yang 2004 *rptd. in* Qin *et al*. 2005). Population genetics, species migration, gene conservation, and tree improvement strategies have been studied using polymorphic isozymes, RAPDs, and SSRs (Huang *et al.* 1998; Fineschi *et al.* 2000; Seabra *et al.* 2000; Aravanopoulos *et al* 2005). Genetic transformations have been successfully

achieved in *C. sativa* using plasmids carried by *Agrobacterium tumefaciens,* Smith and Townsend (Seabra and Pais 1999a; Seabra and Pais 1999b; Corredoira *et al.* 2005)

Mapping efforts within the genus have been relatively limited. The first published mapping study used 196 polymorphic isozyme, RFLP, and RAPD markers for a population of *C. dentata* x *C. mollissima* F2 progeny to produce a map containing 12 linkage groups (Kubisiak *et al.* 1997). This map provided 75% genomic coverage across 530 cM using the Kosambi mapping function (Kosambi 1944; Kubisiak *et al.* 1997). Amplified fragment length polymorphisms (AFLPs) were generated by Clark *et al.* (2001) for the same progeny set used by Kubisiak *et al.* (1997), and the previous map was expanded by 21 cM using an additional 275 AFLP markers. In 2005, 24 SSR and 5 small subunit ribosomal RNA (SrDNA) markers were added to this same map (Sisco *et al.* 2005).

Maps of *C. sativa* have been generated using SSR, inter-simple sequence repeats (ISSRs), RAPD, and isozyme polymorphisms. Pseudotestcross mating designs allowed maps to be created for each of four parents used in these studies (Casasoli *et al.* 2001; Casasoli *et al.* 2004). Each map contained between 108 and 187 markers, covered between 720 and 848 cM, Kosambi, and provided 68 to 84% coverage (Casasoli *et al.* 2001; Casasoli *et al.* 2004). A recent study demonstrated that 11 of the 12 linkage groups from Casasoli *et al.* (2001) showed synteny with 11 groups from the most recent *C. dentata* x *C. mollissima* map (Sisco *et al.* 2005).

Studies on trait inheritance of chestnuts have yielded important progress as well. Often, morphological and resistance traits appear to be controlled by one or few genes. For instance, in interspecific crosses, vein hair density, twig hair density, and green or red stem color have been each respectively assigned to control by a single putative locus, while interveinal leaf hairs and male sterility each appear to be dominated by two loci and stamen types are putatively controlled by four loci (Kubisiak *et al.* 1997; Bolvanský and Mendel 1999; Soylu 1992 *rptd. in* Bolvanský and Mendel 1999). Multiple other traits among various chestnut species and cultivars have been studied using quantitative genetics. Traits of interest have

included survival, vigor, nut quality, tree form, morphology, and blight resistance (Botta et al. 2005; Craddock *et al.* 2005; Alvarez *et al.* 2005). So far, only one study has attempted to use RAPD markers to map disease resistance in *C. sativa* to root rot caused by *P. cinnamomi*, but the results of this study were inconclusive (Seabra 2000).

1.4. Conclusion

Clearly, the effects of *P. cinnamomi* on American chestnut are quite severe. Wherever it is present, this pathogen could easily undermine the re-establishment of blight-resistant American chestnut. The effects of *P. ramorum* on American chestnut are less well defined, but the imminent and potentially rapid spread of this disease throughout the range of American chestnut warrants concern that this pathogen will also threaten The American Chestnut Foundation's success.

Efforts to reintroduce American chestnut to the southeastern forests of the United States are currently in a precarious position, as the Foundation's focus on threats from *P. cinnamomi* and *P. ramorum* is relatively new. Relatively little work has focused on *C. dentata* or on its resistance to *Phytophthora* spp.; molecular marker research concerning the disease association between these two species appears to be lacking. This thesis is part of an initial effort to investigate the genetic basis of resistance of the interspecific cross *C. dentata* x *C. mollissima*. to *P. cinnamomi* and *P. ramorum.*

This study seeks to provide a basis for continued investigations through a series of controlled-inoculation trials and through the development of a genetic linkage map. Chapter 2 investigate several of the Foundation's breeding lines with respect to their resistance to root rot caused by *P. cinnamomi* in controlled inoculation trials, and their reaction levels when inoculated with *P. ramorum.* The results of these trials are discussed in light of determining the mode of inheritance and the number of loci controlling resistance to each disease. The genetic linkage map presented in Chapter 3 was developed for an F1 individual from the *C. mollissima* 'Mahogany' line ('KY115'), which is an important breeding line held by the Foundation.

The purpose of the map is to provide a basis for future correlations between genotypic and phenotypic data derived from progeny of 'KY115' challenged with *P. cinnamomi* in controlled inoculation trials that are currently underway. Conclusions drawn from this and future studies will help provide the Foundation with the knowledge needed to build an efficient and successful breeding selection program that encompasses resistance to both *P. cinnamomi* and *P. ramorum.*

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Table 1.1 Selected literature reporting mortality of *Castanea* spp. caused by *Phytophthora cinnamomi* in controlled inoculation trials

Reference	Inoculation Method	Species	Susceptibility/ Mortality (%)[a]
Milburn and Gravatt 1932[b]	seedling	*C. dentata*	72[c]
	various: stem and soil	*C. sativa*	74
		"southern chinkapins"	67
		C. crenata	3
	"larger plants"	*C. mollissima*	0
	various: stem and soil	*C. sativa*	17
		C. crenata	5
		C. mollissima	2
Dugelay 1933 (*rptd. in* Day 1938b)	grafted rootstock	*C. sylvestris microcarpa*	NR
Day 1938b	stem inoculations	*C. sativa*	25
		C. crenata	80 [s]
			75
Crandall *et al.* 1945[b]	Seedling inoculation (agar plugs in stems and transplanting seedlings to infected soil)	*C. alabamaensis* Ashe	70
		C. alnifolia Nutt.	82
		C. ashei Sudw.	94
		C. crenata	4
		C. dentata	70
		C. henryi	2
		C. margaretta var *arcuata* Ashe	60
		C. mollissima	1
		C. ozarkensis Ashe	70
		C. pumila (L.) Mill.	74
		C. sativa Mill.	38
	Field tests	*C. senguinii*	0
		C. crenata	0
		C. henryi	0
		C. mollissima	0
		C. sativa	100
		C. senguinii	0
Borrod 1971	Callus tissue	*C. sativa* and *C. sativa* x *C. crenata*	NR
Vieitez *et al* 1982[b]	Seedling (?)	*C. mollissima*	0
		C. crenata	0-38
		C. sativa	91.8-100
		C.s. x *C.m.* or *C.c.*	28-94
Salesses *et al.* 1993	Excised leaves, excised stems, *in vitro* stem tissue, whole-plant	*C. sativa*	various
Robin and Desprez-Loustau 1998[b]	Greenhouse seedling (Excised bark)	*C. sativa*	0- ~60 [s] (6.6 cm lesions)
Rhoades *et al.*2003[b]	Greenhouse seedling	*C. dentata*	0-25
Sanchez *et al.* 2004	Greenhouse seedling	*C. sativa*	26 [s]

[a]Susceptible refers to percentages of killed and unhealthy trees, mortality refers to killed trees. All values refer to mortality figures except where noted by [s] for susceptibility.

[b]Actual percentages vary from reported average mortality depending on the isolate used, as well as other treatments within the study (eg., fungicide, soil compaction, etc.)

Table 1.2 Genomic research in *Castanea* spp.

Reference	Species	Method	Purpose	Number of Fragments[a]	Map Size[b]	Map Coverage[c]	LG[d]	Software[e]	LOD, θ[f]
Seabra *et al.* 1996	*C. sativa*	RAPD	RAPD development	1-2					
Kubisiak *et al.* 1997	*C. dentata x C. mollissima*	isozyme, RFLP, RAPD	morphology and blight resistance	196	530.1	75%	12	Linkage-1 JoinMap 1.1	4 NR
Huang *et al.* 1998	*C. dentata*	isozyme, RAPD	genetic diversity	14-22					
Oraguzie *et al.* 1998	*C.* spp.	RAPD	genetic typing	10 primers					
Botta *et al.* 1999	*C.* spp. and *Q.* spp.	SSR	SSR development	9 loci					
Lang and Huang 1999	*C. mollissima C. seguinii C. henryi*	isozyme	relatedness; genus origin	20 loci; 12 enzymes					
Paffetti *et al.* 1999	*C. sativa*	RAPD	RAPD development	34					
Periera *et al.* 1999	*C. sativa*	isozyme	genetic variation	7					
Seabra and Pais 1999a, Seabra and Pais 1999b	*C. sativa*	genetic transformation	*P. cinnamomi* resistance	2 genes					
Villani *et al.* 1999a	*C. sativa*	isozyme, RAPD	relatedness	11 52					
Villani *et al.* 1999b	*C. sativa*	allozyme	relatedness	16					
Santana *et at.* 1999	*C. sativa, C. crenata*	RAPD	genetic typing	164					

[a] Number of fragments refers to the total number of fragments (RFLP, AFLP) or polymorphic loci (RAPD, SSR, isozyme, allozyme) studied or mapped, except where noted.
[b] Map size refers to the cM distance covered in the map in Kosambi units.
[c] Map coverage takes into account the estimated genome size and the cM covered in the map.
[d] LG refers to the number of linkage groups attained in the map.
[e] Software indicates the different software programs used to construct the map.
[f] LOD (threshold)/ θ refers to those values used to group markers within linkage groups.
NR refers to data that were not reported.

Table 1.2 (cont.)

Reference	Species	Method	Purpose	Number of Fragments[a]	Map Size[b]	Map Coverage[c]	LG[d]	Software[e]	LOD, θ[f]
Fineschi *et al.* 2000	*C. sativa*	cpRFLP	species migration	3					
Seabra 2000	*C.* spp.	RAPD	genetic diversity and *P. cinnamomi* resistance	29 primers					
Valdiviesso *et al.* 2000	*C. sativa*	RAPD	relatedness	125					
Casasoli *et al.* 2001	*C. sativa*	ISSR, RAPD, isozyme	adaptivity	187 ♀ 148 ♂	720 ♀ 721 ♂	76%♀ 68% ♂	12	Mapmaker 2.0 JoinMap	4 .4
Clark *et al.* 2001	*C. dentata x C. mollissima*	isozyme, RFLP, RAPD, AFLP	morphology and blight resistance	356	551	NR	13 +2 pairs	JoinMap 2.0	NR NR
Connors *et al.* 2001	*C. dentata*	cDNA library	GenBank comparison	50 clones					
Gualão *et al.* 2001	*C. sativa*	RAPD, ISSR	relatedness	235					
Connors *et al.* 2002	*C. dentata*	mRNA analysis	cystatin coding	1					
Alvarez *et al.* 2003	*C. sativa*	proteins: SDS-Page	genetic diversity	35					
Buck *et al.* 2003	*C. sativa*	SSR	SSR development	8 loci					
Fu and Dane 2003	*C. pumila*	allozyme	genetic diversity	11 enzymes					
Marinoni *et al.* 2003	*C. sativa*	SSR	SSR development	24 loci					
Yamamoto *et al.* 2003	*C. crenata*	SSR	genetic typing	14-16 loci					

[a] Number of fragments refers to the total number of fragments (RFLP, AFLP) or polymorphic loci (RAPD, SSR, isozyme, allozyme) studied or mapped, except where noted.
[b] Map size refers to the cM distance covered in the map in Kosambi units.
[c] Map coverage takes into account the estimated genome size and the cM covered in the map.
[d] LG refers to the number of linkage groups attained in the map.
[e] Software indicates the different software programs used to construct the map.
[f] LOD (threshold)/ θ refers to those values used to group markers within linkage groups.
NR refers to data that were not reported.

Table 1.2 (cont.)

Reference	Species	Method	Purpose	Number of Fragments[a]	Map Size[b]	Map Coverage[c]	LG[d]	Software[e]	LOD, θ[f]
Barreneche *et al.* 2004	*C. sativa and Q. robur*	SSR	comparative mapping	4 (*C. sativa*) 15 (*Q. robur*)	NR	NR	7 +2 pairs	Mapmaker 2.0 JoinMap 1.1	6 NR
Casasoli *et al.* 2004	*C. sativa*	ISSR, RAPD, isozyme, SSR	adaptivity and drought resistance	109 ♀ 108 ♂	848.6 ♀ 832.9 ♂	84% ♀ 75% ♂	12	Mapmaker 2.0	3 .28
Aravanopoulos *et al.* 2005	*C. sativa*	ISSR, isozyme	genetic conservation	73, 16					
Aravanopoulos and Drouzas 2005	*C. sativa*	isozyme	genetic typing	9 loci					
Yang *et al.* 2004	*C. mollissima*	RAPD, isozyme	genetic diversity	20					
Beccaro *et al.* 2005	*C. sativa*	SSR	genetic typing	7 loci					
Botta *et al.* 2005	*C. sativa*	SSR	genetic characterization	10 loci					
Bounous *et al.* 2005	*C. sativa*	SSR	genetic typing	7 loci					
Corredoira *et al.* 2005	*C. sativa*	genetic transformation	plasmid transfer	NA					
Costa *et al.* 2005	*C. sativa*	SSR	genetic typing	5 loci					
Martín *et al.* 2005a	*C. sativa*	SDS-page (proteins and albumins)	genetic variation	35					
Martín *et al.* 2005b	*C. sativa*	SSR	genetic typing	6 loci					
Kubisiak and Roberds 2005	*C. dentata*	RAPD, SSR	genetic diversity	25					
Sisco *et al.* 2005	*C. mollissima* x *C. dentata*	AFLP, SSR, SrDNA, isozyme	genetic mapping, blight resistance	275, 24, 5, 1					

[a] Number of fragments refers to the total number of fragments (RFLP, AFLP) or polymorphic loci (RAPD, SSR, isozyme, allozyme) studied or mapped, except where noted.
[b] Map size refers to the cM distance covered in the map in Kosambi units.
[c] Map coverage takes into account the estimated genome size and the cM covered in the map.
[d] LG refers to the number of linkage groups attained in the map.
[e] Software indicates the different software programs used to construct the map.
[f] LOD (threshold)/ θ refers to those values used to group markers within linkage groups.
NR refers to data that were not reported.

CHAPTER 2
Leaf and Whole-plant Responses of *Castanea* spp. to *Phytophthora cinnamomi* and *Phytophthora ramorum*

Research with chestnut species indicates that the Chinese chestnut, *Castanea mollissima* Blume, is resistant to root rot caused by *Phytophthora cinnamomi* Rands, while the American chestnut, *Castanea dentata* (Marsh.) Borkh. is highly susceptible (Crandall *et al*. 1945). A new pathogenic threat for American chestnuts has developed more recently with the introduction of *Phytophthora ramorum,* Werres, De Cock, and Man in't Venld., into the United States (Hansen *et al.* 2005). Because of its high aggressiveness against other members of the Fagaceae family, it is possible that *P. ramorum* may also cause severe disease and mortality in infected American chestnut. The American Chestnut Foundation is preparing to deal with *P. cinnamomi*, and perhaps *P. ramorum*, by selecting for resistance against these pathogens in their current breeding lines. To conduct this selection process, it is advisable to investigate the basis for genetically controlled resistance. This chapter seeks to provide an initial investigation into the principles associated with such a selection program.

The objective of this chapter to is investigate the mode of inheritance and the number of loci in Chinese and American chestnuts controlling resistance to *Phytophthora cinnamomi* and responses to *Phytophthora ramorum*. To meet these objectives, mortality or leaf symptoms from controlled inoculation trials will be compared to predicted Mendelian segregation patterns. Because pathogen resistance in forest trees can be conferred by a single dominant gene (Wilcox *et al.* 1996), the question of interest is whether or not there is statistically significant evidence to suggest that the observed patterns break from this assumption.

2.1. Root Inoculations

Introduction of a pathogen to soil or artificial media is a reliable way to conduct controlled inoculation trials (Dhingra and Sinclair 1995). Root inoculation procedures for testing plants for resistance to *P. cinnamomi* under local fieldhouse conditions are well established (Benson *et al*. 1998; Hinesly *et al.* 2000;

Frampton personal communication), and were used as guidelines for the following series of root inoculation trials.

2.1.1. Materials and Methods

Three seasons of testing were performed using the root inoculation method described by Hinesly *et al.* (2000). A pilot screening trial was conducted in 2003, using pure Chinese and American chestnuts in addition to hybrids and backcrosses between these species. The purpose of this trial was to determine if the procedures and testing conditions would produce results that agreed with previous studies (Milburn and Gravatt 1932; Crandall *et al.* 1945) indicating that *C. mollissima* was resistant to *P. cinnamomi* and that *C. dentata* was susceptible. A second trial was conducting in 2004 using a third generation backcross family [B3 = ((F1x American) x American) x American] which contained a greater number of samples. During 2004, The American Chestnut Foundation bred two first-generation backcross families (B1 = F1 x American). In 2005, these two B1 families were used in a series of four *P. cinnamomi* root inoculation trials.

2.1.1.1. Pilot Screening Trial of 2003

This trial utilized 192 seedlings from 13 seedlots of various breeding lines and hybridization levels of Chinese and American chestnuts. (Table 2.1) Seeds were provided by Drs. Hebard and Sisco of The American Chestnut Foundation from crosses bred in 2002. The inoculated material included: 41 American chestnuts, 36 Chinese chestnuts, 4 interspecific hybrids (F1), 14 first-generation backcrosses, and 81 hybrid backcross progeny (B1xB1=B1F2). Several lines of *C. mollissima* were represented, including 'Nanking' (Gr119 in an F1 cross), 'Glen' (GL444 in a B1 cross), and 'Meiling' (TM287, TM441, and TM482 in B1-F2 crosses). All other seedlots were open-pollinated Chinese or American chestnuts, and were assumed to be pure species.

All seeds were packed in peat, sprinkled lightly with water, and stored at 4˚C in Ziploc bags (S.C. Johnson and Son, Racine, WI), which were punctured to allow for airflow. The seeds were stratified from September 2002 until March 2003.

The plants were grown, inoculated, and assessed by members of the Christmas Tree Genetics program (CTG) at North Carolina State University in 2003. Seeds were sown March 28, 2003 in individual Anderson bands (0.768 m^3, 0.047 ft^3) (Anderson Die and Manufacturing, Portland, OR). The medium was a peat:perlite:vermiculite mixture (1:1:1 by volume), with a supplement of 59 ml (0.25 cups) of lime for each 34 L (36 quarts) of medium (The American Chestnut Foundation recommendations). Planting depth was 2.5-3.8 cm (1-1.5 inches) below the surface of the medium. Each plant received a tag with the family and tree number (within family), and all plants were placed in a greenhouse, where natural day length was supplemented with high-intensity metal halide lamps at an intensity of 95.8 μmol/m^2/s (Spero Electric Corporation, Cleveland, OH) for a total day length of 16 hours. The temperature was maintained at about 24˚C during the day and 15-18˚C at night. Seedlings were watered as needed and were fertilized *via* a Dramm Syphonject brass siphon mixer (Dramm Corporation, Manitowoc, WI) with 15-16-17 Peat-Lite fertilizer containing micronutrients (J.R. Peters, Inc., Allentown, PA). The fertilizer was applied every 1-2 weeks according to the following schedule: 150 ppm N on April 3, 50 ppm N on April 17, 200 ppm N on April 30, 150 ppm N on May 9 and 16, and 100 ppm N on May 29.

Each family was divided in half and grouped in trays for use in one of two replications. Seedlings were arranged randomly within each replication. Within a tray, Anderson bands containing seedlings were spaced to alternate with empty bands to avoid crowding. Poorly growing or heat damaged seedlings were eliminated from the study. On May 29, two individuals from each of the three B1-F2 families were removed to serve as controls in a separate tray for each replication, for a total of 12 control specimens. All plants were then transferred outdoors to the shaded NCSU Horticulture Field Labs phytopathology pad for inoculation.

The *P. cinnamomi* culture selected was a single zoospore isolate, 23SS04, originally isolated from Fraser fir, *Abies fraseri* (Pursh) Poir. (Drs. Benson and Grand, Plant Pathology, North Carolina State University). Inoculum was prepared by transferring and growing plugs from stock cultures maintained by Dr. Benson in

the center of a Petri plate containing corn meal agar (CMA; Becton Dickinson, Sparks, Maryland) and supplemented with pimaricin (2 mL/L), ampicillin (250 mg/L), rifamyacin (2 ml/L), pentachloronitro-benzene (PCNB; 125 mg/L), and hymexazol (50 mg/L) (Kanwischer and Mitchell 1978). These additional ingredients (PARPH) are selective for the growth of *Phytophthora* spp. and were added before the medium was autoclaved at 121˚C and 1.03 x 10^5 PA for 20 minutes on a liquid cycle. After several days of growth on CMA-PARPH, 8 mm mycelial plugs were taken from actively growing colonies, and transferred to CMA for more vigorous growth for several days. Flasks of inoculated rice grains were prepared according to the following procedure. Twenty-five grams of enriched long grain rice (Riviana Foods, Inc., Houston, TX) were placed in 17 ml diH_2O in a 250 ml flask, covered tightly with foil, and autoclaved at 121˚C and 1.03 x 10^5 PA for 20 minutes on a liquid cycle. The contents of the flask were then stirred to ensure the grains were separated, re-autoclaved, and shaken to separate the grains again. Fifty 4-mm mycelial plugs were transferred to each flask from the edges of actively growing colonies. The flasks were then placed in a dark incubator at 25˚C and shaken daily to ensure maximum colonization of the rice grains by *P. cinnamomi*. After 10-14 days of incubation, five rice grains were randomly selected from each flask, plated on CMA, and examined under a light microscope to verify the presence of the *P. cinnamomi* on the rice grains. In this and all other trials, colonies of *P. cinnamomi* were present on all the examined rice grains.

Two replications of chestnuts were inoculated five weeks apart on June 3 and July 8, respectively, using the following procedure. A glass stirring rod was used to create three holes approximately 2.5 cm deep in the growing medium; these holes were equidistant between the plant stem and the side of the Anderson band. Two colonized rice grains were placed in each of the holes, covered with medium, and tamped lightly. Except for the separate trays of non-inoculated controls, each plant received a total of six colonized rice grains.

All trays, including the non-inoculated controls, were placed directly on a covered gravel surface of the shaded phytopathology pad. Seedlings were irrigated twice daily for about 30 min with approximately 1.8

cm (0.7 in) of water *via* impact head rotating sprayers for the duration of the trial. The seedlings were also exposed to an unusually high amount of natural rainfall throughout the summer of 2003.

Symptoms were assessed and recorded on a bi-weekly schedule by the CTG program for 16 weeks. Assessments were based on the percent of the shoot that was necrotic: 0 (completely healthy), 20, 40, 60, 80, and 100% (seedling was dead). Because the last assessments were made in October, some doubt existed as to whether the appearance of decline was due to *Phytophthora* susceptibility or seasonal senescence; therefore, any seedlings that were suspected to be alive were retained through the winter in a greenhouse and re-assessed in a single follow-up observation in April 2004.

2.1.1.2. B3 Screening Trial of 2004

Control-pollinated seeds were provided by Dr. Hebard and the Kentucky Chapter of The American Chestnut Foundation from control-pollinated crosses bred in 2003. Families included a third generation backcross (B3) from the *C. mollissima* 'Graves' line, 2 F1 families, and an open-pollinated *C. dentata* seedlot. (Table 2.1)

Seed stratification, medium preparation, planting technique, plant spacing, and labeling were conducted as described for the trials in 2003. The same greenhouse was used to house germinating seeds. Seeds were sown randomly within family groups on March 16, 2004. From April 21 to May 27, seedlings were hardened off in afternoon shade and fertilized weekly with 100 ppm N (same fertilizer formulation and delivery system used in 2003). On June 9, controls were established by randomly selecting and isolating several trees from each family in a separate tray. Seedlings were then transported to the same testing site used in 2003. This year, however, trays were placed on Ray Leach tube trays (22.86 cm (9 in) high; Steuwe and Sons, Inc., Corvallis, OR) to prevent direct contact with the ground.

Inoculum was prepared and root inoculations were preformed on June 2 using methods described for the 2003 trials, except that a total of two rice grains were used for each plant, with only one grain per hole.

Plants were assessed weekly by the author using the rating scale described for the 2003 trial. Except for the non-inoculated control specimen, all live plants were re-inoculated on July 29 (eight weeks after the initial inoculation event). Plants that were assessed as dead were discarded after September 16, and the remaining plants were transported indoors to a greenhouse for overwintering as in 2003.

2.1.1.3. B1 Screening Trials of 2005

Control-pollinated seeds were provided by Dr. Hebard of The American Chestnut Foundation from crosses bred in 2004. Seedlots included two B1 families from the 'Mahogany' line, an F1 family, an open-pollinated *C. dentata* family and an open-pollinated *C. mollissima* seedlot. (Table 2.1) Seeds were stratified using previously described methods from September 2004 until January 4, 2005. Upon removal from stratification, seeds were stored at room temperature for four days before planting. Planting medium, planting depth, plant spacing, and tags were handled according to the methods described for previous trials. All seeds and families were randomly distributed without replication.

Seeds were sown in the same greenhouse and under the same conditions as in 2003 and 2004. Three weeks after sowing, all plants were moved to a greenhouse which was maintained at 22±5°C. A maximum-minimum thermometer placed at bench height, was examined daily, and yielded a regular daily high *ca.* 21°C in the first greenhouse, and 26.5°C in the second greenhouse.

Seedlings were watered as needed and were supplemented with 5-16-17 Peat-Lite fertilizer containing micronutrients as in 2003 using the same fertilizing system. Fertilizer was applied every 1-3 weeks according to the following schedule: 25 ppm N January 15, 50 ppm N January 22, 75 ppm N January 29, 100 ppm N February 19, 150 ppm N February 26, and 200 ppm N March 8. Application of 200 ppm N fertilizer solution appeared to burn the leaves, so 100 ppm N was subsequently used on March 14 and 21.

Leaf tissue from each plant was collected from the youngest, most succulent leaf showing the least amount of burn (where applicable) and stored in individually labeled bags (10.2 x 15.2 cm^2 (4 x 6 in^2), 4 mil,

Associated Bag Company, Milwaukee, WI) for DNA preservation. Leaf tissue for genotyping parental trees was mailed overnight from the Foundation's farms in Meadowview, Virginia, in March 2005. Tissue was stored briefly at 4°C until DNA extraction was performed (Chapter 3), and at –80°C long term.

2.1.1.3.1. Spring Greenhouse Inoculation Trial #1

In early March, fresh cultures of *P. cinnamomi* isolate 23SS04 were obtained from Dr. Benson's stock cultures and transferred to CMA-PARPH plates from his lab. Black specks, a contaminant, were noted throughout the medium within three days after transfer; advice suggested this contamination would not complicate the inoculation trial. Rice-grain inoculum was prepared as described for the screening trials of 2003 and 2004.

Plants were moved to the Department of Forestry greenhouse facilities at Method Road on March 28, and were maintained at approximately 19-22°C for optimal pathogen growth. Trays of plants in Anderson bands were placed on Ray Leach tube trays, which were laid in a basin built with lumber and pond lining on top of the bench. The basin was designed to catch irrigation water and collect it in an inflatable pool underneath the bench; collected water was treated with sodium hypochlorite (about 10% v/v commercial bleach:collected water) and allowed to sit for a day before emptying.

Root inoculations were performed on March 29 using 2 infested rice grains per plant according to the methods used in 2004. Specimens were watered daily by hand to the point of run-through until an automated system was installed on April 10. At this time, standard weighted drip emitters (M.L. Irrigation, Laurens, SC) were installed in each Anderson band. This system provided a flow-through rate of approximately 10% by delivering approximately 100-110 ml of water per plant per irrigation event. The system was run approximately 3-4 times per day and was controlled by a Campbell Scientific 21X Data Logger (Logan UT) using input from CS615 soil moisture reflectometers (Campbell Scientific, Logan, UT). The system was programmed to prevent a subjectively determined level of desiccation on the upper surface of the medium. This schedule was continued until July 5.

2.1.1.3.2. Spring Greenhouse Re-inoculation Trial #2

Leftover rice grains from the initial inoculation event were used for reinoculation after several grains were plated out and actively growing, non-contaminated cultures were verified for all of the plated grains. All living inoculated plants were reinoculated on May 29 (eight weeks after the original inoculation event) using previously described methods, except that a total of three rice grains per plant were placed in three holes 4 cm deep. Twenty seedlings from two first generation Japanese backcross families [(F1 x *C. dentata*) x *C. dentata*] which had been received from Dr. Sandra Anagnostakis, Connecticut Agricultural Experiment Station, were added to the study. (Table 2.1) These were planted in Anderson bands on April 2, and except for five seedlings selected for use as controls, were inoculated on May 29 with the other seedlings. Two larger *C. crenata* seedlings were received and planted during the week of April 21 and were used as additional control plants.

2.1.1.3.3. Summer Lathhouse Re-inoculation Trial #3

On July 5, all specimens were transported to the lathhouse located behind the Plant Pathology shaded pad that was used in the 2003 and 2004 trials. Plants were maintained on Ray Leach tube trays to prevent contact of the medium and containers with the ground. Irrigation occurred twice daily via impact head rotating sprayers that delivered approximately a total of 110 ml water in two applications per day.

Fresh inoculum was prepared from stock cultures, and inoculation was repeated on July 24 (eight weeks after the prior inoculation event and sixteen weeks after the initial inoculation) using the same methods described for previous trials, except that three inoculated rice grains were placed at a depth of 2.5 cm (one per hole). Because response levels in the inoculated material had been minimal, two large Fraser fir seedlings potted in Anderson bands were added to the inoculated material as positive controls, and a third was held as a negative control specimen. The two fir seedlings were inoculated and assessed along with the chestnuts using the previously described procedures and rating scale.

2.1.1.3.4. Fall Greenhouse Re-inoculation Trial #4

To prevent the seedlings from entering dormancy, the seedlings were re-invigorated by adhering to the following regime of intensive care (Hebard, personal communication). On August 22 (four weeks after inoculation), all plants were removed from the lathhouse and placed in full sun to promote shoot growth and, more importantly, new root growth which would hopefully provide new sites for infection. Once the growing medium was allowed to dry, water-soluble Miracid Soil Acidifier Plant Food 30-10-10 with micronutrients (Miracle-Gro, Marysville, OH) was applied according to following regime: 780 ppm N until run-through for three days, 260 ppm N until run-through for 12 days, and 520 ppm N with less water for 7 days. On September 14, most plants were beginning to break bud and all were returned to the greenhouse at Method Road and watered as needed while leaf expansion continued. High-intensity metal halide lights (Spero Electric Corporation, Cleveland, OH) were installed and used at an intensity of 130.1 $\mu mol/m^2/s$ to supplement natural day length for a total of 16 hours in order to prevent dormancy and continue the experiment through the fall.

The weighted drip emitters used in the spring trials was assumed to create a conical zone of moisture within each Anderson band, and may have adversely affected the survival of the inoculum in the uppermost portion of the medium. To avoid repeating this situation, a new irrigation system was designed using Shrubbler® 360° adjustable flow spray stakes (Antelco Corporation, Longwood, FL). Spray heads were installed for each plant and adjusted to limit delivery to their respective containers. The new irrigation system provided eight points of irrigation within the approximately 51.6 cm^2 (8 in^2) of medium surface area within each Anderson band. This approach was used to overcome the conical zone of available moisture that may have existed in the indoor trials inoculated in March and May. The daily amount of irrigation water delivered (approximately 220 ml) was roughly half the amount received in the spring, and approximately twice the daily amount in the summer.

When nearly all trees had developed new whorls of leaves, fresh inoculum was again prepared from stock cultures of isolate 23SS04, as described previously. Except for the controls, all living plants were re-

inoculated on October 15 (12 weeks after the prior inoculation event and 28 weeks after the original inoculation). A total of three rice grains were place in each container, with one grain in each of three holes that were 4 cm deep.

Plants were watered heavily on the day of inoculation and irrigation was controlled thereafter by the use of a Campbell Scientific 21X Data Logger for a timed delivery of approximately 110 ml per seedling twice daily, in the morning and in the evening. As in the spring trials, the amount of water delivered was designed to maintain a subjectively determined moisture level at the surface of the medium. Mortality was assessed weekly until December 11 (eight weeks after reinoculation and 36 weeks after the initial inoculation). At that time, the experiment was terminated.

On October 27, several fir specimens growing in Ray Leach tubes were added to the experiment (Table 2.1) Six fir families from susceptible species were represented as follows: three seedlings from a single family of Fraser fir, *Abies fraseri* (Pursh) Poir., twelve seedlings from a single family of Guatemalan fir, *Abies guatemalensis* Rehder, nine seedlings from three families of balsam fir, *Abies balsamea* (L.) P. Mill., and three seedlings from a single family of bracted balsam fir, *Abies balsamea* var. *phanerolepis,* Fern. These seedlings were retained in their Ray Leach tubes and used as checks to see if *P. cinnamomi* was pathogenic. Each treated seedling was inoculated with one colonized rice grain and placed in Ray Leach tube trays next to the chestnuts in the greenhouse. Five additional seedlings from the Fraser fir family were transplanted into Anderson bands already containing inoculated chestnuts. Controls were established using three additional Fraser fir seedlings, one balsam fir seedling, and one bracted balsam fir seedling.

2.1.2.4. Isolation of Phytophthora cinnamomi *from root fragments*

Root samples were harvested from dying plants to determine the cause of death. Samples were preferentially collected to include an interface of living and dead tissue, which should indicate pathogen advancement within the root. Roots samples were rinsed in tap water, blotted with paper towels, and plated on a *Phytophthora* selective medium (PARPH) made of either CMA or potato dextrose agar (PDA; Becton

Dickinson, Sparks, Maryland). No samples were taken in 2003 trials, but samples were taken from approximately 35% of dying samples in 2004 and approximately 95% of dying samples in 2005. Samples were also taken from both of the large inoculated Fraser fir seedlings used in the outdoor summer trial #3 of 2005. Successful reisolation of *P. cinnamomi* from these specimens was interpreted to mean that the specimen was dying from disease induced by *P. cinnamomi.*

2.1.2.4. Recapturing Phytophthrora cinnamomi *from the growing medium*

Because of a low incidence of disease, attempts were made to isolate *Phytophthora cinnamomi* from the growing media of healthy plants in 2005 according to the methods described by Ferguson and Jeffers (1999). The purpose of the isolations was to determine whether the inoculum was still alive in the growing media of seedlings that could theoretically be susceptible to disease caused by *P. cinnamomi* based on their parental heritage. Therefore, healthy inoculated American chestnut or backcross seedlings were chosen for sampling. On June 29, eight plants were randomly chosen and tested, and on September 29, five different plants were randomly selected and tested.

2.1.2.6. Statistical Analysis of the Root Inoculations

The mortality rating for each seedling was transposed into binary code by seedlot. Seedlings rated as 100% necrotic were categorized as "dead" and were given a value of 100, and all living seedlings were categorized as "alive" and given a value of 0. Chi-square goodness-of-fit tests were performed on the mortality percentage for each seedlot using SAS software (version 8.0, SAS Institute, Inc., 2001, Cary, NC). When the null hypothesis was no segregation, a Student's t-test was performed when the observed pattern did not perfectly meet the predicted ratios. The null hypothesis was that the observed value did not differ from the expected values, and all tests were considered significantly different at $p \leq 0.05$; evidence suggested that the null hypothesis should be rejected in such cases. (Table 2.1)

The expected inheritance patterns for each seedlot were determined according to Mendelian inheritance patterns for a dominant gene of major effect at a single locus controlling resistance. The Chinese chestnut

parents were hypothesized to be homozygous dominant (RR) for resistance and their open-pollinated progeny were expected to show a segregation pattern of 1:0 (alive:dead). The F1 hybrid Chinese x American seedlings were expected to be heterozygous dominant for resistance and would therefore exhibit a resistance level that was identical to their Chinese parent. The American chestnut parents were assumed to be homozygous recessive (rr) and the expected ratio for their open-pollinated progeny was 0:1. The expected ratios for other families are as follows: 1:1 for B1 crosses, 0:1 or 1:1 for B3 crosses, and either 3:1, 1:1, or 0:1 for crosses between first generation backcrosses (B1-F2).

2.1.2. Results and Discussion

2.1.2.1. Pilot Screening Trial of 2003

The American chestnut seedlots consistently showed mortality when exposed to *P. cinnamomi,* and while a few survived the 16-week trial, none of the initial survivors was alive in April 2004. (Table 2.1) Neither the Chinese seedlings nor the F1 hybrid (Gr119xKH2uu) showed any mortality. Mortality in other hybrids or backcrosses was variable, ranging from 65% for the most resistant B1-F2 family (TM287xTM441) to 78.5% for the B1 family (AM440xGL444) and approximately 100% for the other B1-F2 families (TM441xTM482 and TM482xTM441). Three of the twelve control specimens were also dead at the end of the trial.

There was no significant difference in mortality between June and July inoculations. At the mid-way point in both replications (week 8), most data suggested the observed mortality values followed predicted Mendelian inheritance patterns for a single, dominant resistance gene. Two B1-F2 crosses showed little mortality at week 8, but at week 16, they conformed to the expected ratio (0:1).

A final assessment of the supposed survivors in April 2004 suggested that the assessors may have been correct to assume that the symptomatic appearance of some of the surviving trees late in the season (where assessment scores < 100%) was due to seasonal senescence rather than infection by *P. cinnamomi* (data not shown). The decision to accept or reject the null hypothesis was the same in the week 16 and the spring

assessments. The only difference was that the single survivor from the 'Lake Logan Intersection' American chestnut family at the end of week 16 died during the winter.

A potentially significant, complicating factor in this trial may have been the amount of water the plants received; while maintaining a high moisture content in the medium is desirable for encouraging the growth of *P. cinnamomi*, it is possible that the amount of water the plants received from rain events and irrigation combined was excessive for encouraging healthy plant growth. A second complicating factor was the death of 3 of the 12 controls. This mortality may have been caused by placing the Anderson band trays directly onto the ground, which, while covered with gravel and a thick-mesh tarp material, may have harbored *P. cinnamomi* isolates from concurrent and prior trials. Such contamination may have hampered the effort to limit the inoculum to a single source and decreased the certainty that mortality for the inoculated trees was caused solely by *P. cinnamomi* isolate 233SS04. Attempts to isolate *P. cinnamomi* from root samples of dead control seedlings were not performed, but isolations from control seedlings of Fraser fir that died in a concurrent study were confirmed positive (Frampton personal communication).

Other conclusions regarding species and cultivar resistance can also be drawn. Obviously, wild *C. dentata* populations do not carry resistance, while populations of *C. mollissima* do. The 'Nanking' and 'Glen' cultivars clearly demonstrated resistance in the F1 and B1 crosses, respectively. Resistance in the 'Meiling' appears to have been retained in TM287, but not in other parental material. This is not unexpected since no selection for resistance had been made in the B1 parent material.

Overall, the data from the 2003 trial appear to indicate that resistance may follow patterns associated with a single dominant gene. American chestnuts appear to have the susceptible genotype rr, and Chinese chestnuts appear to have the resistant genotype RR. F1 hybrids are phenotypically resistant, and would theoretically have a genotype Rr, where the R allele conferred by the Chinese parent is dominant. The B1 crosses involving trees AM40xGL444 were phenotypically resistant; the F1 parent GL444 would appear to be associated with an Rr genotype. The progeny crosses of TM441 and TM482 are not statistically

different from a 0:1 segregation pattern, and would therefore appear to have susceptible rr genotypes. Because of this, and because progeny from the cross TM482xTM441 segregated in a pattern that was not statistically different than 1:1 at $p < 0.05$, it may be assumed that all resistance observed in this B1-F2 family was conferred by TM287 and that TM287 has a genotype of Rr.

It may be, however, that there are one or more resistance genes of minor effect corresponding with delayed mortality. This could help explain why progeny of the cross TM441xTM482 did not conform to expected mortality values until week 16, while other susceptible families mostly conformed by week 8. While the results of this trial may be skewed due to rather small sample sizes for several seedlots (e.g., four F1 individuals and two Andrea Lee open-pollinated Chinese chestnut families), it would appear that the 2003 screening test indicates that resistance to *P. cinnamomi* within *C. mollissima* follows the patterns suggested by a dominant gene at a single locus, with possible genes of minor effect at other loci.

2.1.2.2. B3 Screening Trial of 2004

No mortality occurred in the control plants or the F1 hybrids throughout 2004. All of the inoculated American chestnuts died, and 84.6% of the B3 population died by the end of the trial, and this differed from the expected 1:1 segregation ratio at $p \leq 0.0001$. Attempts to isolate *P. cinnamomi* from the roots were positive, indicating that this pathogen was the cause of mortality.

The survival of the 2004 controls, compared to some mortality in the 2003 controls, may indicate the importance of avoiding direct container-to-ground contact in order to preclude pathogenic contamination from previous experiments. However, the use of a B3 population in which selection for *P. cinnamomi* resistance had not been made in the parental material reduced the chance for resistance to be demonstrated. Only 14% of the B3 seedlings survived the 2004 trial season, and all survivors were relatively small specimen that were no more than 15.2 cm (6 in) tall. Only a single survivor was present after re-inoculation in 2005.

This high mortality rate suggests several hypotheses. First, embryonic lethal alleles or viability alleles (Hedrick and Muona, 1990; Kuang *et al.*, 1999; Nikaido *et al.*, 2000) may reduce the number of resistant progeny and therefore affect resistance segregation patterns. Second, there may be several genes of minor effect that delay the susceptible mortality response. This theory could be supported by Mason (1912) and Butterick (1913), who documented delayed mortality in wild American chestnut stands when exposed to *P. cinnamomi*. Third, the 'survivors' may have been escapes and the expected ratio should have been 0:1 (Mason 1912). This theory could be supported by the fact that the remaining B3 seedlings were all relatively small compared to the rest of family, and would therefore have had fewer root tips to serve as an infection court. An additional point of support for this is that the observed mortality ratio after the second season was 99%, which would have met the expected ratio of 0:1 at $p < 0.0001$. Regardless of the cause for high mortality, the results indicate the importance of including *P. cinnamomi* resistance in a selective breeding strategy. Disease resistance controlled by a single dominant gene can easily be lost after four generations of breeding without appropriate selective pressure.

2.1.2.3. B1 Screening Trial of 2005

No mortality occurred in the control specimens or in the Chinese, American, or F1 chestnut seedlings throughout the 2005 testing season. Three inoculated seedlings were lost due to an irrigation failure for a single plant or due to the chestnut blight; these specimens were removed from the data set. Only 12 plants (24%) from the cross KY117xWB348 and 7 plants (7.3%) from the cross KY115xWB348 showed mortality by the end of the year after four inoculations under various environmental conditions. (Table 2.2) While both of the larger Fraser fir specimens from the summer trial were dead by August, none of the fir specimens in Ray Leach tubes and none of the specimens planted as companions to chestnuts in Anderson bands showed any symptoms of susceptibility eight weeks after inoculation when the trial was ended.

The pathogen was isolated from the roots of dead plants, indicating that *P. cinnamomi* was the cause of mortality. To determine whether the pathogen was present in the growing medium of healthy specimen that were assumed to be susceptible, soil recaptures were performed at the end of June and again in late

September. All samples produced positive results, indicating that the pathogen was present in the growing medium.

Steps were taken to ensure that the inoculum source was a pure culture of *P. cinnamomi.* In preparation for each inoculation event, rice grains were plated out and scanned under the microscope for the uncontaminated presence of *P. cinnamomi.* In addition, rice grains were plated out from each flask used in the July inoculations and the resulting cultures were inspected by Dr. Benson. The result was a positive identification of pure cultures of *P. cinnamomi* growing from the rice grains.

Extreme care was taken to provide the most disease-conducive conditions in the final trial in October 2005. First, a new irrigation delivery system was installed to avoid limited moisture availability within each Anderson band. Second, the amount of water delivered was comparable to that provided in previous outdoor trials. Third, the plants had been under a fertilizer regime which produced actively growing root tips the entire length of the root ball; this was verified by non-destructively randomly selecting and removing at least twenty-four plants from their containers and inspecting the root balls for new growth. While more disease occurred in this trial compared to the previous three trials, there was still little disease response, and no mortality was observed in the American chestnuts, which were expected to show a response of 100% mortality.

Several causes of disease failure can be theorized. One hypothesis is that the use of two inoculated rice grains was insufficient to cause disease. Six rice grains were used in 2003 and results in that year most closely met the expected hypotheses for segregation within each seedlot. However, the dramatic results of 2004 using only two rice grains clearly shows that the use of two grains of inoculum is sufficient to cause disease. Increasing the number of grains used per plant from two grains in the March inoculation to three grains in subsequent inoculation events still failed to produce a reasonable disease response even in the American chestnut seedlings. This suggests that a lack of mortality was not related to the number of grains used.

To evaluate the pathogenicity of isolate 23SS04, a subsequent trial with an independent set of two American chestnut families is currently underway. For this trial, approximately 125 seedlings from open-pollinated *C. dentata* families were inoculated with isolate 23SS04 and ten isolates generously donated by Dr. Benson, Plant Pathology, North Carolina State University, and Dr. Jeffers, Plant Pathology and Physiology, Clemson University. These trials were inoculated with procedures and conditions identical to those of 2003. Preliminary data show that the isolate 23SS04 caused 80% mortality four weeks after inoculation (Frampton, personal communication), suggesting the isolate had not lost pathogenicity.

Another hypothesis is that the environment or host plant development was unfavorable for disease development. This theory is supported by a lack of response in both American chestnut and fir specimen. Throughout all of the trials, none of the American chestnut seedlings suffered mortality. While the two Fraser fir inoculated in Anderson bands in the summer trial were dead by week 14, none of the smaller fir specimens used in the October trial, including those used as companion plants within the Anderson bands containing chestnut specimens, showed mortality by week 8 in the fall experiment. According to Benson, *et al.* (1998), percent mortality for these species should approach 100%. This lack of response indicates an unfavorable environment for disease development.

Any one of several conditions could have created an unfavorable condition for disease development. One hypothesis is that the plants had ceased growing new root tips, which are a targeted infection site by *P. cinnamomi* (Zentmeyer 1980; Gow 2004). This theory is significantly weakened by the fact that seedlings of 2005 were approximately 11 and 19 weeks old when inoculated in March and July, respectively; in comparison, plants were 10 and 18 weeks for the 2003 replications and 12 and 20 weeks old during the inoculation events of 2004. Because disease development was successful in similarly aged plants during other years, it is unlikely that the age of the seedlings was the cause of a failure for disease development during the spring and summer 2005 inoculation trials.

An unfavorable environment may have been caused by a suboptimal watering regime. During the first 10 days after the initial inoculation in March, when only one heavy watering event was applied each day by hand, the moisture content of the medium might have been too low in the upper portion of the medium within each Anderson band due to warm temperatures in the greenhouse. Likewise, the drip irrigation design used in the March and May inoculations may have only produced a conical distribution of water in the medium, which still allowed the upper inch or so of medium to become too dry. On the other hand, the automated irrigation system used in the spring trials provided approximately 4 times as much water compared to the amounts delivered in the 2003 and 2004 trials.

It is not possible to determine the cause of the minimal disease development in the 2005 trials. Under the appropriate conditions, susceptible American chestnuts exposed to *P. cinnamomi* may develop disease symptoms and die in a matter of a few weeks, as demonstrated by the results of 2003 and 2004. Thus, some environmental or developmental condition must have inhibited disease during the 2005 trials. It is also possible that a combination of factors prevented disease development, or that different conditions inhibited disease development during each inoculation attempt.

2.1.3. Conclusions

From the data generated in 2003, it appears that genetic control against *P. cinnamomi* may be provided by a dominant gene of major effect inherited from *C. mollissima* in a single locus. This is demonstrated by three breeding lines that are held by The American Chestnut Foundation and used in their breeding program to incorporate blight resistance. The pilot trials of 2003, however, also highlight the need for an experimental design that precludes contamination issues. The death of controls and the small sample sizes of various seedlots caused some degree of uncertainty in interpreting the results.

The B3 screening trial of 2004 demonstrated eliminated the problem of mortality of control specimens, but it also highlighted the pitfall of using advanced generation material in which no selection has been made for

resistance against the pathogen of interest. However, results from this trial did not contradict the hypothesis that resistance is controlled by a single dominant gene.

The trials of 2005 did not proceed as anticipated, as a greatly reduced expression of disease was observed. The lack of mortality in American chestnuts 36 weeks after inoculation is a new phenomenon and may indicate disease failure due to host development conditions, or more likely, environmental conditions. It is therefore unlikely that the low degree of mortality in other seedlots was due to genetic resistance. Future disease assessments for this collection of plant material will be pursued and will hopefully further our knowledge regarding the genetic control of resistance to *P. cinnamomi* in chestnuts.

2.2. Leaf Symptomology Assay in Chestnut Selections Challenged with *Phytophthora cinnamomi*

Because the *P. cinnamomi* root inoculation trials of chestnut material in 2005 were generally uninformative, another effort was made that year to investigate the reactions of that material to *P. cinnamomi.* These efforts used excised leaves from specimens that were still being tested using root inoculation methods. The objective of this study was to determine whether leaf spot symptoms (if any) could be correlated with theoretical expectations for root rot disease in seedlots challenged with *P. cinnamomi*. (Table 2.1) The leaf assay was designed according to the methods of Salesses *et al.* (1993) for inoculating and evaluating excised leaves of European chestnut, *Castanea sativa* Mill., challenged with *P. cinnamomi.*

2.2.1. Materials and Methods

Before running the *P. cinnamomi* leaf assay on the full set of available chestnut host material, the following procedures were tested on leaves from two seedlings each of *C. dentata, C. mollissima,* and an F1 hybrid in two sequential trials. The first trial differed from later methods in that it lasted only 9 days and was located in the lab, and the plates were not sealed with parafilm so that the filter paper support for excised leaves could be watered twice daily with 1 ml of diH_20. The second trial was performed using methods identical to those described below.

Some of the plants used for the 2005 root inoculation trials were used for the large excised leaf assay. (Table 2.3) Leaves from 124 B1 plants from two families were tested: 83 from family KY115xWB348 and 41 from family KY117xWB348. Additional leaves from 8 American, 10 Chinese, and 6 F1 hybrid chestnuts were also used. Two replications were conducted, with one leaf used in each replication to represent each tree. A tree was omitted from either the entire study or the second replication as necessary to ensure that at least one leaf remained on the plant that had been newly induced by the fall fertilizer applications. This allowed susceptible symptoms to be readily demonstrated in the ongoing root inoculation trial.

According to Salesses *et al.* (1993), the young leaves showed higher rates of necrosis and larger necrotic responses caused by *P. cinnamomi* than older adult leaves. Thus, leaves collected for the current study were chosen based on their stage of maturity, with a preference for leaves that had nearly or recently reached full expansion (Stein and Kirk 2003). Additionally, each leaf was given two grades, based on age and color, in order to provide a basis for statistical comparisons. Age grades ranged from 1 to 5 and included the following categories: 1—immature, 2—at the point of maturity, 3—healthy and fairly recently mature, 4—mature, but more aged, 5—old leaf showing definite signs of aging or senescence. Leaf color was also assessed, to account for additional undetermined variations in leaf appearances; these differences were likely caused by variation in leaf age, seedling nutrition, or both. Leaf color grades were 1—normal, green, 2—red, or green tinged with red, and 3—yellow.

Round sheets of 90 mm diameter filter paper (Whatman Company, Floram Park, NJ) were placed in the lids of plastic Petri dishes (90 mm lid diameter). Leaves were cut in half and, when necessary, the apical portion was trimmed so that the remaining leaf section would fit into the lid of the Petri dish. Each leaf half was surface-sterilized by immersion in a 0.5% sodium hypochlorite in diH_2O (10% v/v commercial bleach solution) and rinsed three times in diH_2O (Stein and Kirk 2003).

Fresh transfers of *P. cinnamomi*, isolate 233SS04, were made from cultures already grown for three days in a dark incubator at 25°C on plates of PARPH media made with a V8 base (Campbell Soup Co., Camden, NJ). Non-inoculated plates of V-8-PARPH were held under the same conditions as control plates. The upper surface of each leaf-half received one plug (4 mm diameter) from the agar plates of control medium, and five plugs of agar, containing mycelium, collected from the growing edge of the inoculated plates. Three equidistant plugs were placed to the left of the leaf's midrib and two were placed on the right, with the control plug in the bottom right corner of the leaf. The plugs were preferentially placed between the lateral veins and approximately half-way between the midrib and the margin. This allowed two control events and ten inoculation events for each seedling in each replication.

In the second replication, carborundum powder was used as an abrasive on one half from each leaf. The leaf half (distal or apical) which received the carborundum treatment was chosen randomly for each tree. The powder was applied to the leaf half with a fine paint-brush in order to cause light wounding of the epidermal tissue, thereby allowing inoculum to penetrate older leaves more easily.

After inoculation, each plate received approximately 1 ml of diH_2O from a clean spray bottle and was sealed with parafilm to maintain high humidity. Plates were incubated in a walk-in growth chamber, which was maintained at 23°C with 16 hour days under two fluorescent lights per shelf (28.7 μmol/m^2/s, Wide Spectrum for Planet and Aquarium, GE, Fairfield, CT). Plates within each replication were placed randomly on one of eight shelves in three separate benches. The first replication was set up on November 11, and the second replication was set up on November 12.

Responses to each inoculation and control event were measured multiple times within the 11-day observation period, and all responses were measured on the 11th day for each replication. The maximum and minimum linear distances were measured to the nearest 0.0254 mm (0.001 in) were taken for each necrotic spot associated with an agar plug, and a subjective grade was given to each spot indicating the relative severity of the reaction ("spot-grade"). The spot-grades were according to the following scale: 0—

no reaction, 1—large necrotic reaction overtaking a significant portion of the leaf, 2—contained necrotic reaction, 3—limited reaction mainly within the veins (the major veins turned dark but the interveinal areas were relatively unaffected), 4—possible prior leaf condition, 5—possible bruising due to carborundum wounding, 6—the agar plug was dislocated, 7—mold growth, and 8—faint spot of uncertain cause.

Analysis of the minimum and maximum reaction measurement, the reaction area index (minimum reaction measurement multiplied by the maximum measurement) and reaction type were averaged across the 10 inoculation points for each leaf tested. Mean reaction values for each tree and seedlot were calculated and analyzed using SAS statistical software, based on maximum reaction measurements. An ANOVA conducted with age as a covariable and using family and whether or not the tissue was wounded with carborundum powder as class variables.

2.2.2. Results and Discussion

Both of the mini-trial leaf assays produced results consistent with expectations for *P. cinnamomi* resistance in Chinese and American chestnuts and F1 hybrids. (Table 2.1) In the first trial, the leaves from *C. mollissima* seedlings showed no reaction to the inoculum, the younger of the F1 leaves showed a single pronounced grade 2 reaction near one inoculated agar plug, and the American leaves showed multiple grade 2 reactions under 50% of the inoculated agar plugs, while the Chinese chestnut leaves again showed no reaction. In the second trial, the younger of the two American leaves showed grade 2 responses under 75% of the inoculation events, and only one grade 2 reaction was seen on the younger of the F1 leaves. Because of the greater sensitivity of the younger leaves, it was determined that younger leaves (just reaching or just past the point of reaching maturity) were the ideal age for testing.

For the larger trial containing two replications, only 10 of the 480 non-inoculated controls (2%) showed a reaction after 11 days of incubation. Because of this uniformity, all control events were excluded from statistical analysis. Locations which had been inoculated and which did not fit into the spot-grade

categories of 0, 1, or 2 were also omitted from analysis in order to avoid complications in attributing necrosis to a susceptible response.

None of these variables analyzed in the full ANOVA model were significant at $p < 0.1$. This was true regardless of whether the dependent variable was the average minimum reaction measurement, the reaction area, or reaction type. When using the full ANOVA model with the maximum reaction measurement as the dependent variable, the only variable showing a significant effect at the $p < 0.1$ level was the family variable. However, this model only explained 19.4% of the variation in reaction values, according to the R^2 estimate.

2.2.3. Conclusions

The results from the small trials suggest that the amount of leaf necrosis in assays may be correlated with theoretical expectations (listed in Table 2.1) for resistance to root rot disease caused by *P. cinnamomi*, but this was not confirmed in the larger trial. The significance of the family origin in the full ANOVA model is interesting, but its overall importance in the results is offset by the low R^2 for the model. As Salesses *et al.* (1993) concluded, leaf symptomology assays involving *P. cinnamomi* may be feasible but are not the best indicators of response to *P. cinnamomi* due to large amounts of variability that cannot be accounted for by the family origin. The experimental error of the larger trial in this study was too great to yield any firm conclusions regarding differences in the size of lesions caused by *P. cinnamomi* on the leaves of various American and Chinese chestnut families and crosses. Future trials of this nature should seek to capture a more uniform stage of leaf development and better control of interfering environmental variables.

2.3. Leaf Symptomology Assays in Chestnut Selections Challenged with *Phytophthora ramorum*

Various methods have been used to investigate resistance to sudden oak death and dieback disease induced by *P. ramorum* in 77 woody species, including some oaks trials (Denman *et al*. 2005a; Hansen *et al*. 2005). These studies have shown that, in general, for mature woody species, the cumbersome procedures of cutting and inoculating log sections provide the most accurate correlations to natural symptoms of infection

in the field. For testing smaller plants, the best indicator of field reactions is achieved through whole-plant immersion of seedlings in a zoospore suspension; the second best method, at least for some species, is to inoculate the main stem or a branch. Excised leaf inoculations are most useful as a rapid test for general susceptibility (Hansen *et al.* 2005). Zoospore concentration, leaf age, and leaf origin within the crown may all affect the results of *in vitro* leaf inoculation trials (Denman *et al*. 2005a; Hansen *et al*. 2005). For this experiment, the only method available was the leaf assay, as the material needed to be preserved for other experiments.

2.3.1. Materials and Methods

Three *P. ramorum* leaf assays were conducted using two sets of material. (Table 2.3) The spring and fall assays were performed using the remaining samples from the previously described *P. cinnamomi* root inoculation trials. The summer assay was conducted using leaves from a different set of materials, which provided a larger number of *C. dentata* seedlots; this trial was conducted to establish a more appropriate leaf age for testing and to study the heritability of necrotic reactions to *P. ramorum*.

2.3.1.1. B1 Screening trial – Spring

Plants were grown as described for the *P. cinnamomi* root inoculation trial of 2005. See Table 2.3 for the number of leaves from each family or seedlot that were used. Leaves were harvested and mailed on April 5, 2005 to Dr. Susan Diehl, Department of Forest Products, Mississippi State University, for collaborative testing. Because of previous fertilizer burns to the leaves and because of the advanced age of the leaves, it was difficult to obtain a sample set of standardized leaves that were close to the point of reaching maturity. However, the youngest, most healthy leaf was harvested in all cases. Upon arrival in Dr. Diehl's lab, leaves were stored at 4°C for several weeks until they could be assigned to one of four consecutive treatment workdays.

Eight or nine 10 mm disks were taken from each leaf, and were surface sterilized by dipping into a 0.6% sodium hypochlorite solution for 2 min, followed by three sterile water rinses. Five to six disks from each

leaf were immersed for 1 min in a suspension of zoospores of four *Phytophthora ramorum* isolates: Pr-143 from *Quercus agrifolia* Née, BS-77 from *Lithocarpus densiflora* (Hooker and Arnold) Rehder, Pr-108 from *Umbellularia californica* (Hooker and Arnold) Nutt., and Pr-52 from *Rhododendron* sp. These isolates are rated as having a moderate aggressiveness (Hüberli *et al.* 2005; Ivors *et al.* 2004). Three additional disks from each leaf were dipped into a water control.

After immersion in the zoospore suspension or water control, disks were placed on wet filter paper in a Petri dish and held in a dark incubator at 20°C. Reaction scores were assessed after 7 days on the following scale of 1-11: 1—no disoloration (green leaf), 2—brown edges (about 10% of the leaf was a dead brown, necrotic color), 3—10-30% necrosis, 4—30-50% necrotic, 5—60% necrotic, 6—70% necrotic, 7—80% necrotic, 8—90% necrotic, 9—100% of the leaf is greenish brown, 10—100% of the leaf is necrotic, 11—100% black-brown. Scores of 9 were designated for disks from leaves that were noticeably older, and the discoloration appeared to be due to senescence, rather than necrotic lesions.

Reisolations were performed on approximately 10% of the inoculated disks. For this process, leaf disks were surface-sterilized again in a weak bleach solution and plated on a selective medium, CMA-PARP (Kanwischer and Mitchell 1978). Cultures were held in a dark incubator at 20°C and inspected after one week for the growth of *P. ramorum*.

2.3.1.2. American trial from rooted cutting stock –Summer

Material for this experiment included seeds from six wild *C. dentata* crosses, and full-sib progeny from one controlled cross. (Table 2.3) All *C. dentata* parent trees were located in the wild in the western corner of Virginia, near Meadowview, Virginia. Samples were also taken from five progeny from an open pollinated seedlot of *C. mollissima*, one *C. crenata* individual, and five B1 individuals ((Japanese x American) x American); the Japanese chestnut seedlings were grown as described in the materials and methods for the 2005 *P. cinnamomi* root inoculation trial. (Table 2.1)

The seeds were received on July 15, 2004 and sown as part of a rooted cutting study on July 20, 2004. They were originally planted in Anderson bands (1.21 L), and were transplanted after four weeks to 7.57 L (2-gal) Tall Treepots™ (Stuewe and Sons, Corvallis, OR) on August 17, 2004 in order to promote less restricted growth during the first season. The medium was the same as described for the *P. cinnamomi* screening trial, except for the incorporation of 118.3 ml (0.5 cup) Controlled Release 18-6-12 Osmocote™ (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) per 34 L (36 quarts) of medium; a top-dressing of 30 ml (2 tbsp) Osmocote™ was also applied to the plants in early February 2005. The plants were maintained in a greenhouse and were generally managed to produce continuous growth. Conditions measured at benchtop height were warm (~35°C maximum in the summer, ~14°C minimum in the winter,). Long day lengths (16-20 hr) were maintained using high-intensity metal halide lamps operating at an intensity of 95.8 μmol/m^2/s (Spero Electric Corporation, Cleveland, OH). Specimens were watered, staked, hedged, and treated as needed for spider mites with Talstar™ (FMC, Philadelphia, PA) and for fungal gnats with Gnatrol™ (Abbott Laboratories, Chicago, IL).

One whole young leaf from the top of each plant was harvested for testing on July 19, 2005. Prime leaves consistently nearing maturity were easily selected. Each leaf was placed in an individually labeled bag (10.16x15.24 cm^2 (4x6 in^2), 4 mil, Associated Bag Company, Milwaukee, WI) for overnight transport to Dr. Diehl.

Each leaf was assigned to one of three inoculation times: one within a few days of shipment, one approximately two weeks after shipment, and one approximately three weeks after shipment. Disks were taken from each leaf, surface sterilized and treated either with a zoospore suspension or water control; four to five disks were treated as controls, and five to six disks were inoculated, using the same four sources of *P. ramorum* as described previously. An additional set of disks from two families, American family 'G' ((PL14V x OP) and the Chinese open-pollinated family, were set up as before, except that for these samples disks were dipped in a 70% ethanol solution for 60 seconds rather than in sodium hypochlorite.

Incubation conditions were the same as described earlier, and reactions on all disks were assessed seven days after inoculation. After inoculation or exposure to the water control, all disks were placed on wet, sterile filter paper in a Petri dish and held in a dark incubator for 7 days at 20°C. Reaction of the leaf disks were assessed on the same scale as before, except that the score of 9 was not needed, since these leaves were not as old as the sample set used in the spring. Therefore, scores of 10 and 11 became scores of 9 and 10, respectively. There were no reisolations performed on this set due to time restrictions.

2.3.1.3. B1 Screening Trial – Fall

The plants were grown as described for the *P. cinnamomi* root inoculation trials of 2005. The number of the specimens used per seedlot is shown in Table 2.3. Leaves were harvested and mailed to Dr. Diehl on October 11, 2005. Because of the recent flush of growth caused by an intensive fertilization regime, it was easier to collect a standard leaf age than it had been in the spring.

Each leaf was assigned to one of three treatment days: October 14, 15, or 17. The leaves were sterilized and controls were established as described previously. Inoculations were performed with only one of the four isolates used in the previous trial, BS-77 from *L. densiflora*. For each leaf, three to five disks were used as controls, and four to eight disks were inoculated. Incubation conditions were the same as described earlier, except that assessments were taken on both the 7^{th} and the 14^{th} day after inoculation. Reactions were assessed using the 1-10 scale as described for the summer assay. Reisolations were performed on approximately 80% of the inoculated disks.

2.3.1.4. Statistical Analysis of the Phytophthora ramorum *Leaf Symptomology Assays*

For the spring trial, a score of 9 was used to designate the greenish brown reaction color which was thought to be caused by oxidative browning; during statistical analysis all scores with a value of 9 were converted to values of 1 (no disease reaction). No other scores were converted in this or other trials.

During the fall trial, disks from younger leaves occasionally produced a dark brown, water-soaked appearance, which may have been in reaction to the sterilization process (Susan Diehl, personal communication). These disks were eliminated from the data set during statistical analysis.

Analyses of reaction scores from each assay were performed with SAS statistical software. Individual tree means and family means were calculated. An ANOVA model was used to detect seedlot differences in inoculation scores based on the effect of family, inoculation score, and control score. A Tukey-Kramer separation of least square means test was run at the $p \leq 0.05$ level to determine significant seedlot differences within each trial.

Variance component estimates were obtained for the open-pollinated American chestnut families using restricted maximum likelihood (REML) techniques. These components were used to calculate individual tree and family mean heritability as follows: $h_I^2 = 4\ (\sigma_f^2)\ /\ (\ \sigma_f^2 + \sigma_E^2)^2$ and $h_F^2 = (\sigma_f^2)\ /\ (MSF / k)$; where h_I^2 is the individual tree heritability (narrow sense), h_F^2 is the family mean heritability (σ_f^2) is the family variance, (σ_E^2) is the total variance, MSF is the expected means square value family, and k is the coefficient of σ_f^2 in the MSF.

The variance and standard error for the heritability estimates were calculated using the Dickerson approximation as follows: $V(h_I^2) = 4\ (\sigma_f^2)\ /\ (\ \sigma_T^2)^2$ and $V(h_F^2) = (\sigma_f^2)\ /\ (\ \sigma_f^2 + {}^1/_5\ \sigma_E^2)^2$; where $V(h_I^2)$ is the variance of the individual tree heritability and $V(h_F^2)$ is the variance of the family mean heritability, and (σ_T^2) is the total phenotypic variance.

2.3.2. Results and Discussion

2.3.2.1. *BC1 Screening trial – Spring*

Based on results from various ANOVA models, the best separation of mean seedlot reaction scores was obtained by treating seedlot and the inoculation day as class variables, and the control scores as a covariable. The results showed that seedlot, inoculation day, and average control score all had significant

effects on the inoculation score for each tree at the $p \leq 0.005$ level. This model explained 62% of the variation in the reaction scores.

Least squares mean (LSMean) for reaction scores were calculated for each seedlot. (Table 2.4) The American chestnuts ranked as the least reactive seedlot, with an LSMean of 6.4 (on the 1-11 scale). The F1 family and the B1 family KY117xWB348 followed at 7.0 and 7.8, respectively. The open pollinated Chinese chestnut seedlot and the B1 family KY115xWB348 ranked as the most reactive material and had LSMeans of 8.0 and 8.6, respectively. The Tukey-Kramer separation of means test only yielded a significant difference between the American and KY115 x WB 348 family at the $p < 0.05$ level. (Table 2.4)

The general age and condition of the leaves harvested for this trial, and their being held in an incubator for several weeks before inoculation likely had a negative impact on the results and reliability of this trial. Dr. Diehl noted that many of the control disks turned brown, and that these false positives made it difficult to assess whether browning in the inoculated disks was a reaction to inoculation or if was caused by oxidative browning caused by phenolic compounds within the leaves. It was therefore concluded that the trial should be repeated when a more standardized leaf age could be collected and when the collaborative testing between campuses was more coordinated.

2.3.2.2. *American trial –Summer*

No significant differences between sterilization procedures (bleach as compared to alcohol) were noted during the assessment period. Because of this, disks sterilized with alcohol were included during statistical analysis.

The open pollinated Japanese chestnut seedlings ranked as the least reactive material, with an LSMean of 1.1, which is essentially no reaction on the 1-10 scale. The Japanese B1 chestnut families ‘cross 9’ and

'cross 5' had scores of 3.0 and 4.5, respectively. The open pollinated Chinese chestnut seedlot ranked between the Japanese B1 chestnut families with a score of 3.4. The American chestnut seedlots ranged between 4.1 and 10.0, and were generally the most reactive material, with the exception of the PL14VxOP family which was ranked as less reactive than the Japanese B1 chestnut family 'cross 5.' (Table 2.5)

A Tukey-Kramer separation of means test yielded several interesting comparisons. (Table 2.5) In particular, the open pollinated Japanese chestnuts yielded the lowest reaction level. However due to the limited sample size (n=2), it was not statistically different from some seedlots that were more reactive. The seedlot of Chinese chestnuts showed an intermediate reaction levels between the two Japanese backcross families and was significantly different from five of the American chestnut families. Similarly, the Japanese 'cross 9' was significantly different from the five most reactive Amercian families. Family 'G' (PL14V x OP) was the most resistant of the American chestnut families.

Since the plants used in this experiment were maintained for continual growth and the desired leaf age was more readily available, the material harvested for this trial was better standardized by age. This yielded a more reliable assessment and analysis than could be achieved during the spring trial.

Oxidative browning was much less problematic during the assessment of reactions in this assay than it had been during the spring. An Anova test provided evidence for significant seedlot effects on the inoculation score at $p < 0.01$, but no evidence that control scores had a significant effect on the predicted individual tree scores at $p < 0.1$. This model, where seedlot origin was the only independent variable, explained 42% of the variation in the average inoculation score for each tree.

Statistical analysis of the six open-pollinated American chestnut families yielded a family variance component of 2.69 (family 'A' was omitted from analysis because it was control-pollinated). The individual tree (narrow sense) heritability was 0.30, with a standard deviation of 0.55. The family

heritability was 0.80, with a standard deviation of 0.49. These heritability estimates suggest that reaction scores of leaves from chestnut specimens exposed to *P. ramorum* are under some degree of genetic control.

2.3.2.3. *B1 Screening Trial – Fall*

The reaction scores for the fall trial placed the open pollinated Chinese chestnut material as the least reactive material with an LSMean of 2.2, followed closely the Japanese 'cross 9' at 2.5. The LSMeans for the B1 families KY117xWB348 and KY115xWB348 were 3.5 and 4.1, respectively. The F1 family, with only 3 samples, had a value of 4.5, and the open pollinated American chestnut seedlot was the most reactive, with a value of 6.1 (see Table 2.6).

Based on results from various ANOVAs, the best models for individual tree and seedlot mean reaction scores were calculated by treating seedlot origin and the inoculation day as class variables, and the control scores as a covariable; this was true for the scores from each assessment day (7 and 14). The results for the day 7 assessment indicated that the seedlot and inoculation day had a slightly greater effect ($p < 0.0001$) on predicted tree means in comparison to the control scores ($p < 0.018$). Yet, this model only explained 43% of the variation in the average inoculation score for each tree, as demonstrated by the R^2 value. For the day 14 assessment, the variable with the most significant effect was the inoculation day ($p < 0.0001$) followed by seedlot ($p < 0.0002$), and the trees' individual average control score ($p < 0.0010$). The R^2 value was slightly higher for the day 14 assessment, at 0.46.

A Tukey-Kramer separation of means test for day 7 was run at the $p < 0.05$ level. (Table 2.6) The lowest, least reactive mean score was for the Chinese seedlot followed by the Japanese B1 family 'cross 9.' These two seedlots were significantly different from other seedlots. The F1 and American chestnut materials were the most reactive. The Chinese seedlot and the Japanese B1 family 'cross 9'were significantly different from the F1 family and the American family, but were not different from the B1 families.

The only differences described by the Tukey-Kramer separation of means test for the day 14 assessment were that the means for the Chinese seedlot and the B1 family KY115xWB348 were significantly different at $p < 0.05$. (Table 2.7) This would appear to be influenced by sample size.

All families showed a significant correlation between day 7 and day 14 assessments at $p < 0.001$. Materials from KY115xWB348 and KY117xWB348 that were common to the spring and fall trials did not show a significant correlation between the spring assessment and either of the fall assessment days. Only the American and F1 families were significantly correlated between trials at the $p < 0.1$ level, but these comparisons were made with only five and three samples per family, respectively. The lack of similarity in the rankings of families in the spring and the fall trials could be due to the difference in leaf ages between the trials. The fact that four *P. ramorum* isolates were used in the spring, while only one was used in the fall may also affect the ability to compare the two trials. Thus, it is not possible to consider the fall and spring trials as replicates.

2.3.3. Conclusions

A search for evidence of bimodal reaction scores was not successful in any of the *P. ramorum* assays (data not shown); thus, it is impossible to conclude from the current data that reaction levels in each family are controlled by a single gene. The low R^2 values of less than 0.5 for the fall trials suggest the environmental conditions were not stringent enough to provide accurate results, that the control of resistance to *P. ramorum* is not inherited, that multiple genes may be needed to provide protection, or some combination of these factors.

Given the stronger, more evident family differences as indicated by the Tukey-Kramer separation of means tests, the day 7 assessment of the fall trial can be considered to be the most accurate representation of the response of material in the spring and fall. From these results, it is clear that Chinese chestnut is less reactive than American chestnut. The results from the summer trials and the fact that in the fall, more advanced crosses of Japanese backcross families demonstrated less reactivity than Chinese x American

chestnut intercrosses, suggest that Japanese chestnut may be equally or less reactive to *P. ramorum* than Chinese chestnut. The inability to discern a bimodal pattern in the reaction scores of any given B1 family may suggest that resistance to *P. ramorum* in chestnuts is not controlled by a single dominant gene. Before such a conclusion can be firmly drawn, however, future efforts should focus on reducing the environmental error within the trial, or at least identifying and measuring more variables that may help explain the variation in the results of the trial.

2.4. Future work

Future efforts with *P. cinnamomi* root inoculation trials should focus on attaining reasonable patterns of susceptibility from existing material. Preliminary data at four weeks after inoculation indicate that the isolate has not lost pathogenicity against chestnut. Material remaining from the 2005 trial has been repotted in larger containers of the same type of medium and will be reinoculated with isolate 23SS04 in the spring of 2007 at the outdoor testing location used in previous trials. Mortality of the 2005 American chestnut seedlings will be important as evidence that reasonable diseases responses have been obtained, and hopefully, morality segregation patterns will conform to the theoretical expectations for all seedlots. The expected mortality ratio for the B1 families is 1:1, according to Mendelian principles. If this pattern emerges for the family KY 115 x WB348, additional efforts should focus on mapping the locus controlling resistance on the genetic linkage map developed for this family as described in Chapter 3.

Future efforts in regard to *P. ramorum* should focus on determining the type of effect of this pathogen has on American chestnuts in the field. In an experimental setting, the preferred testing method would be to use a whole-plant dip (Hansen *et al.* 2005). As outlined in Chapter 1, it is possible that American chestnut would not suffer mortality in its role as a host to *P. ramorum*, but that young leaves and branches may become blighted and serve as inoculum reservoirs in the field.

In conclusion, future work should continue to focus on identifying chestnut species and cultivars that demonstrate resistance to *P. cinnamomi* and that are the least reactive to *P. ramorum*; this includes the

identification of cultivars from more advanced generations of breeding and selection. Other objectives should be to determine the mode of inheritance and number of loci controlling resistance or reaction levels. Preferably, these genes of interest would be mapped using co-dominant marker systems, such as simple sequence repeats (SSRs), which are transferable across families and could be used as aids for selective breeding.

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Table 2.1 Results of *Phytophthora cinnamomi* root inoculation trials in 2003, 2004, and 2005 at 16 weeks after inoculation

Year	Seedlot	Seedlings (no)	Observed % mortality	Expected Ratio[a]	χ^2 or t-value	Reject[b]
2003	American: W1	10	100	0:1		
	American: Lake Logan Big Tree	9	100	0:1		
	American: Lake Logan Wood Pile	9	100	0:1		
	American: Lake Logan Intersection	9	88.9	0:1	-1.0[c]	
	American: Edwards Rd, Ashe Co, OP	4	75	0:1	-3.0[c]	
	Chinese: OP	10	0	1:0		
	Chinese: Andrea Lee OP	2	0	1:0		
	Chinese: Edwards Rd, Ashe Co, OP	24	0	1:0		
	F1: Gr119xKH2uu	4	0	1:0		
	B1: AM40xGL444	14	78.6	0:1	-1.9[c]	
	B1-F2: TM287xTM441	23	65.2	1:1	2.1	
	B1-F2: TM441xTM482	49	97.9	0:1	-1.0[c]	
	B1-F2: TM482xTM441	9	100	0:1		
2004	American: Weist 83	6	100	0:1		
	F1: BX172 x A2178	7	0	1:0		
	F1: BX200 x A1345	7	0	1:0		
	B3: Adair Co. Am x GL240	101	84.2	1:1	47.1	****
				0:1	-4.3[c]	****
2005	American: GM Big x OP	6	0	0:1	-∞[c]	****
	Chinese: OP	12	0	1:0		
	F1: VA35 x A1218	5	0	1:0		
	B1[d]: KY117 x WB348	54	24.1	1:1	24.9	****
	B1: KY115 x WB348	96	7.3	1:1	66.7	****
	B1: Japanese 'cross 5'	6	0	1:1	-∞[c]	****
	B1: Japanese 'cross 9'	15	40	1:1	0.6	

[a] Expected Mendelian inheritance ratio (alive:dead) for a dominant gene of major effect at a single locus are as follows: 0:1 for American seedlots; 1:0 for Chinese and F1 seedlots; 0:1 or 1:1 for B1 families; 3:1, 1:1, or 0:1 for B1-F2 families; 0:1 or 1:1 for the B3 family

[b] The number of asterisks indicates the probability of lack-of-fit between the observed and expected segregation patterns. All seedlots met the expected hypothesis, except for those marked with asterisks.
Levels of rejection are as follows: * at $p \leq 0.05$, ** at $p \leq 0.01$, *** at $p \leq 0.001$, and **** at $p \leq 0.0001$.

[c] The Chi-square goodness of fit test is not applicable due to an expected value of zero, so a student's t-test was run instead, and these are the resulting t-values.

Table 2.2 Number of trees showing mortality in each of the root inoculation trials of 2005

			Number dead				Cumulative Mortality[a]	
	seedlot	n (trees)	29-Mar	29-May	24-Jul	17-Oct	# dead	% dead
American:	GMBig x OP	5	0	0	0	0	0	0
Chinese:	OP	10	0	0	0	0	0	0
F1:	VA 35 x A1 218	5	0	0	0	0	0	0
B1:	KY117xWB348	54	0	4	2	7	13	24.1
B1:	KY115xWB348	96	0	1	1	5	7	7.3
B1:	Japanese 'cross 5'	6	NA[b]	0	0	0	0	0
B1:	Japanese 'cross 9'	15	NA	1	2	3	4	40

[a] Percentage of each seedlot assessed as dead 36 weeks after initial inoculation
[b] These families were not included in the test until the May inoculation.

Table 2.3 Number of plants per seedlot inoculated in the excised leaf assays of 2005

Seedlot	*Phytophthora cinnamomi*	*Phytophthora ramorum* Spring	Summer	Fall
American: GM Big x OP[a]	8	7	-	5
Chinese: OP[a]	10	11	-	9
F1: VA35 x A1218	6	5	-	3
B1: KY117 x WB348	41	56	-	41
B1: KY115 x WB348	83	95	-	81
Japanese: OP	-	-	2	1
B1: Japanese 'cross 5'	-	-	5	-
B1: Japanese 'cross 9'	-	-	5	5
American: 'A': CBF2 x MR22	-	-	5	-
American: 'B': CBF2 x OP	-	-	5	-
American: 'C': GM Big x OP[b]	-	-	5	-
American: 'D': GM NewQ x OP	-	-	5	-
American: 'E': GM NewVV x OP	-	-	5	-
American: 'F': HP3 x OP5, OP6	-	-	5	-
American: 'G': PL14V x OP	-	-	5	-
Chinese: OP[b]	-	-	5	-

[a] These seedlots were from 2003 pollinations.
[b] These seedlots were from 2004 pollinations.

Table 2.4 Tukey-Kramer separation of mean seedlot reaction scores for the spring *Phytophthora ramorum* leaf disk assay, at $p \leq 0.05$

Seedlot		No. Seedlings	Mean[a,b]	Comparison[c]
American:	GM Big x OP	7	6.4	a
F1:	VA35 x A1218	5	7.0	ab
B1:	KY117 x WB348	56	7.8	ab
Chinese:	OP	11	8.0	ab
B1:	KY115 x WB348	95	8.6	b

[a] Least Squares Means (LSMeans)

[b] Scores were based on a scale of 1-11:
1: no disoloration (green leaf), 2: brown edges (about 10% was a dead brown, necrotic color), 3: 10-30% necrosis, 4: 0-50% necrotic, 5: 60% necrotic, 6: 70% necrotic, 7: 80% necrotic, 8: 90% necrotic, 9: 100% of the leaf is greenish brown, 10: 100% of the leaf is necrotic, 11: 100% black-brown.

[b] Means followed by the same letter are not statistically different from each other at $p \leq 0.05$

Table 2.5 Tukey-Kramer separation of mean seedlot reaction scores for the summer *Phytophthora ramorum* leaf disk assay, at $p \leq 0.05$

Seedlot	No. Seedlings	Mean[a,b]	Comparison[c]
Japanese: OP	2	1.1	a
B1: Japanese 'cross 9'	5	3.0	a
Chinese: OP	5	3.4	a
American: 'G': PL14V x OP	5	4.1	ab
B1: Japanese 'cross 5'	5	4.5	ab
American: 'F': HP3 x OP5, OP6	5	6.2	ab
American: 'B': CBF2 x OP	5	7.8	b
American: 'C': GM Big x OP	5	8.3	b
American: 'E': GM NewVV x OP	5	8.3	b
American: 'D': GM NewQ x OP	5	9.1	b
American: 'A': CBF2 x MR22	5	10.0	b

[a] Least Squares Means (LSMeans)

[b] Scores were based on a scale of 1-10:
1: no disoloration (green leaf), 2: brown edges (about 10% was a dead brown, necrotic color), 3: 10-30% necrosis, 4: 0-50% necrotic, 5: 60% necrotic, 6: 70% necrotic, 7: 80% necrotic, 8: 90% necrotic, 9: 100% of the leaf is necrotic, 10: 100% black-brown.

[b] Means followed by the same letter are not statistically different from each other at $p \leq 0.05$

Table 2.6 Tukey-Kramer separation of mean seedlot reaction scores for the day 7 assessment of the fall *Phytophthora ramorum* leaf disk assay, at $p \leq 0.05$

Seedlot	No. Seedlings	Mean[a]	Comparison[b]
Chinese: OP	9	2.2	a
B1: Japanese 'cross 9'	5	2.5	a
B1: KY117 x WB348	41	3.5	ab
B1: KY115 x WB348	83	4.1	ab
F1: VA35 x A1218	3	4.5	b
American: GM Big x OP	5	6.1	b

[a] Least Squares Means (LSMeans)

[b] Scores were based on a scale of 1-10:
1: no disoloration (green leaf), 2: brown edges (about 10% was a dead brown, necrotic color), 3: 10-30% necrosis, 4: 0-50% necrotic, 5: 60% necrotic, 6: 70% necrotic, 7: 80% necrotic, 8: 90% necrotic, 9: 100% of the leaf is necrotic, 10: 100% black-brown.

[b] Means followed by the same letter are not statistically different from each other at $p \leq 0.05$

Table 2.7 Tukey-Kramer separation of mean seedlot reaction scores for the day 14 assessment of the fall *P. ramorum* leaf disk assay, at $p \leq 0.05$

Seedlot		No. Seedlings	Mean	Comparison[b]
Chinese:	OP	9	4.9	a
B1:	Japanese 'cross 9'	5	5.1	ab
B1:	KY117 x WB348	41	5.9	ab
B1:	KY115 x WB348	83	7.0	b
F1:	VA35 x A1218	3	8.2	ab
American:	GM Big x OP	5	8.4	ab

[a] Least Squares Means (LSMeans)

[b] Scores were based on a scale of 1-10:
1: no disoloration (green leaf), 2: brown edges (about 10% was a dead brown, necrotic color), 3: 10-30% necrosis, 4: 0-50% necrotic, 5: 60% necrotic, 6: 70% necrotic, 7: 80% necrotic, 8: 90% necrotic, 9: 100% of the leaf is necrotic, 10: 100% black-brown.

[b] Means followed by the same letter are not statistically different from each other at $p \leq 0.05$

CHAPTER 3
Genetic Map for the F1 Parent in a Backcross Family [(*Castanea mollissima* 'Mahogany' x *Castanea dentata*) x *Castanea dentata*] Using AFLP Markers

A considerable body of genomic research has been published for the genus *Castanea*. This work includes several published and unpublished genetic linkage maps using marker systems including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), and amplified fragment length polymorphisms (AFLPs). Progress has also been made with simple sequence repeats (SSRs), expressed sequence tags (ESTs), and inter-simple sequence repeat (ISSR). Relatively little work has been focused on *Castanea dentata* (Marsh.) Borkh., or on its resistance to *Phytophthora* spp., and molecular marker research concerning the disease association of these two species appears to be lacking. This study was part of an initial effort to elucidate the genetic basis of resistance to *Phytophthora cinnamomi* Rands in an interspecific cross *C. dentata* x *C. mollissima* Blume. The objective of this chapter was to use AFLP markers to create a genetic linkage map for an F1 individual 'KY115' (*C. mollissima* 'Mahogany' x *C. dentata* 'RCF1'), which could potentially be used to map the locus(i) controlling resistance to *P. cinnamomi* based on segregation data obtained from root inoculation trials of the original mapping population that will be conducted in the spring of 2007.

~~3.1~~3.1 Materials and Methods

3.1.1. Plant Production

Control-pollinated seeds were provided by Dr. Hebard of The American Chestnut Foundation from crosses bred in 2004. Seeds of a first generation backcross (B1) family (KY115xWB348) were stratified using methods described in Chapter 2. Upon removal from stratification, seeds were stored at room temperature for four days before planting. Planting medium, planting depth, plant spacing, and tags were handled according to the methods described in Chapter 2. All seeds and families were randomly distributed throughout a single replication.

Seedlings were watered as needed and were supplemented with 15-16-17 Peat-Lite fertilizer containing micronutrients as in 2003 using the same fertilizing system. Applications occurred every 1-3 weeks according to the following schedule: 25 ppm N January 15, 50 ppm N January 22, 75 ppm N January 29, 100 ppm N February 19, 150 ppm N February 26, and 200 ppm N March 5. Application of 200 ppm N fertilizer solution appeared to burn the leaves, so 100 ppm N fertilizer solution was subsequently used on March 14 and 21.

Leaf tissue from each plant was collected from the youngest, most succulent leaf showing the least amount of burn (where applicable) and stored in individually labeled plastic bags (10.2 x 15.2 cm^2 (4 x 6 in^2), 4 mil, Associated Bag Company, Milwaukee, WI) for DNA preservation. Leaf tissue for genotyping parental trees was mailed overnight from The American Chestnut Foundation's farms in Meadowview, Virginia, in March 2005. Tissue was stored briefly at 4°C until DNA extraction was performed, and at –80°C long term.

3.1.2. DNA Extraction

Leaf tissue DNA was extracted from 102 samples: 99 progeny samples of the desired B1 family, both parents, and the *C. mollissima* 'Mahogany' grandparent. DNA from 3 progeny samples was contaminated or mislabeled during extraction and were discarded. 25-50 mg fresh leaf tissue were used for each sample. DNA was extracted from each individually stored sample following the QIAGEN DNeasy® 96 Plant Kit protocol (QIAGEN, Venlo, The Netherlands). A modified CTAB method proved necessary in order to obtain DNA from 25 samples which yielded consistently low quantities when using the QIAGEN method. This second method uses the CTAB extraction buffer and procedures as described by Flagel *et al.* (2005), except for the omission of phenol from the phenol+chloroform cleanup solution; it also includes the use of sodium metabisulfite as recommended by Horne *et al.* (2004). Extracted DNA was stored at –80°C long-term, and working dilutions were stored at 4°C.

3.1.3. PCR Reactions, Primer Selection

Amplified fragment length polymorphism (AFLP) reactions were performed on a PTC-100 thermal cycler (MJ Research, Waltham, MA). Restriction digest reactions were performed using 215 ng DNA in 11 µl. 64 samples were processed according to the protocol described by Myburg and Remington (2001), with minor modifications during the restriction digest and adapter ligation steps according to the protocol provided by LI-COR Biosciences with the IRDye™ Fluorescent AFLP® Kit for Large Plant Genome Analysis (LI-COR® Biosciences, Lincoln, NE). Restriction digest and ligation reactions for these 64 samples were performed using the 5x reaction buffer, EcoR1/MSE1 enzyme mix, and adapter mix from LI-COR® Biosciences. T4 DNA ligase from NEB (New England Biolabs, Beverly, MA) yielded a larger quantity of ligated fragments and was therefore used in place of the T4 DNA ligase provided in the LI-COR® kit. Each adapter ligation reaction consisted of 12.5 µl restriction digest mixture, 12 µl LI-COR® adapter mix, 0.35 µl NEB T4 DNA ligase buffer, and 0.15 µl NEB T4 DNA ligase at 400 cohesive U/µl. 37 samples which could not be amplified during the above procedures were processed strictly according to the protocol described by Myburg and Remington (2001).

The preamplification and selective amplification reactions for all samples were done according to the Myburg and Remington (2001) protocol for single dye reactions. Preamplification was performed with Roche® Taq polymerase (Roche Molecular Systems, Inc, Alameda, CA) and an E+1/M+1 primer combination, E+A/M+C. Selective amplification was performed using NEB® Taq polymerase and 32 different E+3/M+3 and E+3/M+4 primers. (Table 3.1) Twelve primer pairs previously used for a *C. mollissima* x *C. dentata* cross were used for selective amplification, which provided approximately 105 markers that could be used as candidates for mapping the F1 parent (Clark *et al.* 2001). In order to reach an arbitrary goal of 200 candidate markers, an additional 73 randomly chosen primer pairs were each screened against a set of 6 samples. The first 20 primer pairs which provided the clearest and most numerous polymorphisms were selected for use against the entire sample population.

Polyacrylamide gels were made with 6.5% KB^{Plus} Gel Matrix (LI-COR®, Inc, Lincoln, Nebraska) for genotyping. Samples were eletrophoresed at 1500 volts for 7 frames on one of three machines (for increased gel capacity). These included a one-dye, model 4000 and 2 two-dye, model 4300 LI-COR® automated DNA sequencers. The 101 samples were divided in approximately half for each primer pair, and were run using a 64-well comb with a ladder standard (50-700bp) on either side of the sample section. After the first samples had run for 7 frames, the comb and gel were reloaded with a second set of samples and 2 ladder standards. This allowed for all samples within a given primer combination to be run within two gels.

3.1.4. Marker Scoring and Analysis

621 polymorphisms from digital AFLP gel images generated by 32 primer combinations were scored for presence or absence using the AFLP-Quantar™ software program (version 1.05; KeyGene Products, Wageningen, the Netherlands). Lane definitions, gel blocks, ladder standards, alignment bands, and link lanes were created as described in the protocol by Myburg and Remington (2001). Because of the number of samples, two gels were run for each primer combination. Polymorphic bands were identified manually and scores were produced automatically by AFLP-Quantar™ software as present (+), absent (-), or undetermined (?) in the first gel. The COPYSCORE procedure was used to automatically select and score bands in the second gel. All sample scores were then examined in the ZOOM window, and in cases of disagreement, visual assessments were used rather than the automated scores.

A quality score was also assigned to each polymorphism based on the clarity of the bands and the ease of scoring . The quality indicators used were, in decreasing order, 'X,' 'Y,' and 'Z.' When a given polymorphism differed in quality between gels, the lower of the two indicators was the one retained.

Raw AFLP data from all 621 polymorphisms from the KY115xWB348 progeny, parents and 'Mahogany' grandparent were scored in AFLP-Quantar™ software. All scores were compiled into a single data set and aligned by sample in EXCEL software (Microsoft Corporation, Redmond, Washington). This data set

was imported and processed with SAS statistical software (version 8.0, SAS Institute, Cary, NC) for data analysis and transformation (Appendix A). Percentages of missing values, parental genotype, and a chi-square goodness of fit test to determine the segregation pattern were examined for each polymorphism. A total of 226 polymorphisms (36.4%) were removed from the data set during statistical analysis (explained below), leaving 395 polymorphisms as candidates for mapping.

The percentage of missing values were determined for each primer pair using SAS statistical software, and for each sample and the data set as a whole using EXCEL software. Of the 621 polymorphisms, 79 (12.8%) were removed from the data set because they contained ≥ 20% missing values. At that point, analysis showed that 12 of the 96 uncontaminated progeny samples contained ≥ 20% missing values due to low quality reactions. These 12 samples were removed from the data set, leaving scores from both parents, the 'Mahogany' grandparent, and 84 progeny samples.

104 polymorphisms (16.7%) were determined to be inherited from the *C. dentata* 'WB348' genotype and to be segregating in a ratio that was significantly different than 3:1 at $p \leq 0.01$; these were removed from the data set, as explained below. Additional polymorphisms which were observed to be inherited from 'WB348' in a nearly 1:1 ratio were omitted during the scoring process, because of potential complications caused during mapping efforts for 'KY115.' 43 polymorphisms (6.9%) were distorted from a 1:1 ratio at $p \leq 0.01$ despite being inherited from the *C. mollissima* x *C. dentata* 'KY115' parent, and these were removed from the data set. Additional polymorphisms showing severe segregation distortion were omitted during scoring, regardless of parental origin. 395 polymorphisms (63.6%) were left as candidate markers for use in mapping.

185 polymorphisms (26.7%) segregated in a ratio that was not significantly different than 3:1 at $p \leq 0.01$, and of these, 155 showed no distortion from a 3:1 ratio at $p \leq 0.05$. All 185 polymorphisms segregating in a 3:1 ratio at these confidence levels were used as candidate accessory markers.

211 polymorphisms (34%) were found that were not inherited from the *C. dentata* 'WB348' parent and that did not segregate in ratios significantly different than 1:1 at $p \leq 0.01$. Of these, 195 showed no distortion from a 1:1 ratio at $p \leq 0.05$, and within this subset, 164 clearly demonstrated inheritance from the F1 parent. All 211 polymorphisms were selected as candidate framework markers.

3.1.5. Map Construction

Candidate framework and accessory markers were coded for map construction using SAS software. Coding for JOINMAP® software (version 3.0, Van Ooijen and Voorrips 2001) was performed using the using the cross-pollinator (CP) population type (heterozygous parents, four possible alleles). Thus, candidates segregating 1:1 were coded as 'lm' (present), 'll' (absent), or '--' (undetermined). Candidates segregating 3:1 were coded as 'h-' (present), 'kk' (absent), or '--' (undetermined). After linkage group assembly, the data were recoded for framework map construction in MAPMAKER™ Macintosh software versions 2.0 and 2.0.68 (E.I. DuPont de Nemours and Company, Wilmington, Delaware). For this program, 1:1 candidate markers were coded as '1' (present), '2' (absent), or '0' (undetermined), and candidate markers segregating 3:1 were coded as '2' (absent), or '0' (either present or undetermined).

All candidate markers were placed into a single data set, imported into JOINMAP® 3.0 mapping software, and processed according to the following process. 164 candidate markers showing a clear inheritance from the F1 parent and no distortion from a 1:1 segregation pattern at $p \leq 0.05$ were used in initial map construction to the exclusion of other candidate markers; linkage groups were established with a minimum LOD score of 5.0. Additional candidate markers were introduced into the data set one by one, and retained if they could be placed within an existing group at LOD = 5.0.

The first group of candidate markers to be added were those that were known to be inherited from the F1 parent and that segregated in a 1:1 ratio at $p \leq 0.01$. The second and third groups included candidate markers with an unknown parental origin that segregated in ratios that were not distorted at $p \leq 0.05$ and $p \leq 0.01$, respectively. These three categories of markers were added, in rounds, so as to capture all cases

where the inclusion of one marker affected the inclusion of any other candidate markers. This was continued until an entire round was achieved in which no more markers were added, at which point all unlinked 1:1 polymorphisms were permanently excluded from the data set. The 3:1 candidate markers were then added in the following order. The first group included candidate markers that were not distorted from a 3:1 ratio at $p \leq 0.05$, and the second group contained those that were not distorted at $p \leq 0.01$. These 3:1 markers were added in rounds until no more markers could be added. This concluded the JOINMAP® software analysis.

Separate data sets were created for each linkage group created during analysis with JOINMAP® software. These data sets were translated into the appropriate code for framework map construction using MAPMAKER™ software. Map distances were estimated using Kosambi mapping function (Kosambi 1944). Because MAPMAKER™ software does not recognize repulsion phase linkages, candidate markers within each linkage group were duplicated and recoded in the opposite phase. Quality indicators were translated to lowercase 'x,' 'y,' and 'z' to indicate the phase change. The files were uploaded to MAPMAKER™ software and maps were constructed using a LOD score of 5.0 and a recombination fraction (θ) of 0.40. Framework map construction with interval support of LOD $\geq$ 3.0 (Keats *et al*. 1991) was completed by ordering each linkage group using the FIRST ORDER command at LOD $\geq$ 5.0, $\theta = 0.4$. Because of the duplicate data sets within linkage groups, this command produced two sequences that were contained identical marker names and orders, but which were present in the opposite phase; the first of the two sequences was the one analyzed. Terminal markers were examined in a LOD table to assess whether they were more strongly associated with an interior marker than their adjacent marker. If this was the case, the terminal marker was removed, and the FIRST ORDER was run on the remaining sequence again. This process was repeated until the terminal markers closest affinity was to the markers next to them. The DROP MARKER command was run to identify markers which would decrease the linkage group by $\geq$ 6 cM when dropped. Markers were dropped individually, and the FIRST ORDER command was run again. The RIPPLE command was also performed to analyze the interval support around each possible permutation of three markers. Those without an interval support at LOD $\geq$ 3.0 were analyzed further, and

one of the markers in the permutation was dropped. This process was repeated until a RIPPLE command yielded interval support at LOD ≥ 3.0 across all permutations. Interval support at LOD ≥ 3.0 indicated that the permutation was at least 1000 times more likely than any other order.

Once the framework map was constructed, 3:1 markers and 1:1 markers that had been removed from the framework map were placed in framework marker intervals using the bin mapping function of MAPPOP software version 0.9 alpha (Vision *et al* 2000). The closest framework marker was recorded for each accessory marker, along with the appropriate bin distance, (that is, the cM distance from the mid-point of the bin to the closest framework marker). . Because MAPPOP software can only place accessory markers within a framework map, markers that were not placed by MAPPOP were evaluated for linkage to terminal markers using the NEAR command in MAPMAKER, and then the LODs command was run to provide the appropriate pairwise distance. Hence, map distances for internal accessory markers were obtained from MAPPOP, and the distances for terminal accessory markers were derived using MAPMAKER software using LODs command.

3.1.6. Estimates of Genome Size and Coverage and Average Distance between Framework Markers

Genome size was estimated according to the method of Hulbert *et al.* (1988) and Chakravarti *et al.* (1991), and modified by Remington *et al.* (1999). The formula employed by Remington *et al.* (1999) was L=[(n(n-1)d)/2k] [1+{1-((2Ck)/n(n-1))1/2}] where L was the estimated genome length, n was the total number of candidate framework markers (regardless of whether or not they were included in the final linkage maps), d was the maximum pairwise map distance between *any* two framework markers (not just adjacent framework markers), and k was the number of pairwise linkages for all (n) 1:1 candidate framework markers (where the minimum LOD score was equivalent to the value used to create the initial linkage groups), and C was the haploid chromosome number. The variables d and k were determined by using the MAPMAKER™ LODS command where the minimum and maximum LODs were 5 and 9999, respectively, and the minimum and maximum θ values were 0.00 and 0.40, respectively.

The average distance between framework markers was determined according to the method described in Remington *et al.* (1999), where the summed distance across all linkage groups was divided by the difference of the number of framework markers minus the number of linkage groups.

3.1.7. Marker Synteny

After the generation of framework maps and the placement of accessory markers, linkage groups from this study were compared with those produced by Clark *et al.* (2001) to investigate marker synteny. This was possible because the populations in both studies shared the same Chinese grandparent, *C. mollissima* 'Mahogany,' and because the maps were for the F1 descendants of this cultivar.

Markers from the primer pairs that were mapped in both studies were listed by linkage group and compared manually between maps. Markers were considered to show synteny between maps if their primer combinations were identical and if they were labeled within 2 bp of markers described by Clark *et al.* (2001).

3.1.8. Marker Origin from the 'Mahogany' Grandparent

The genotype for the *C. mollissima* 'Mahogany' (parent of the F1) was scored in the same gels as the parents and progeny. Table 3.4 presents markers that were absent in the American parent, present or undetermined in the F1 parent, and present in the 'Mahogany' grandparent.

3.2 Results and Discussion

3.2.1. Generation of AFLPs

Initial mapping efforts using only the 12 AFLP primer combinations selected by Clark *et al.* (2001) was assumed to give incomplete coverage, as only approximately 105 polymorphisms were inherited from the F1 parent, regardless of their segregation ratio. 20 additional primer combinations were required to obtain a total 621 polymorphisms scored across 32 total primer combinations. All primers were run against 98 full-sib progeny, both parents, and the *C. mollissima* 'Mahogany' grandparent (other grandparents were of

C. dentata origin, due to the design of the first generation backcross and were no longer available). On average, 19.4 polymorphisms per primer combination were scored across 32 primer combinations. 226 polymorphisms, including all those from the primer combination ACG/CGC and combination AGC/CCA, were removed from the data set during statistical analysis, leaving 395 candidate markers for mapping. This provided an average of 13.2 candidate markers per primer combination across 30 primer combinations.

The total number of polymorphisms generated by primer pair ranged between 8 and 46, and was dependent on the selectivity of AFLP primers. For E+3/M+3 and E+3/M+4 primer pairs, respectively, the average number of scored polymorphisms were 22 and 14.4, and the average number of candidate framework markers were 7.6 and 5.2.

The percentage of polymorphisms that were deleted from the data set due to segregation distortion (6.9%) was less than that reported in previous research within *Castanea* spp. Published distortion levels for various marker types include AFLPs 22%, RAPDs 20%, RFLPs 31%, and isozymes 25% (Kubisiak *et al.* 1997; Clark *et al.* 2001). The relatively low percentage of distorted polymorphisms in this study was likely caused by an effort to avoid scoring markers that appeared to be distorted from a 1:1 or 3:1 ratio.

Polymorphisms segregating in expected Mendelian ratios of 1:1 (indicating inheritance from one parent) and 3:1 (indicating inheritance from both parents) were detected using a chi-square goodness of fit test performed at $p \leq 0.05$ and at $p \leq 0.01$. The null hypotheses were that polymorphisms segregated in patterns that did not differ from a 1:1 and 3:1 ratios, respectively. The increased probability level from 0.05 to 0.01 increased the number of undistorted markers but only affected the final linkage maps by adding 2 framework markers and 18 accessory markers.

3.2.2. Linkage Map Construction

There were 8.8% missing data in the 151 candidate markers used to construct the framework map, ranging from 1 to 31 by sample, and 1 to 16 by primer pair. According to Kubisiak *et al.* (1993), reliable linkage groups and correct marker orders would still be generated by a data set containing this amount of missing data. The inflated percentage of missing values due to the inclusion of markers segregating in 3:1 ratios was not calculated, as these markers were used exclusively as accessory markers and hence did not affect framework map construction.

Initial linkage groups were constructed using JOINMAP® 3.0 where the minimum LOD value was set to 5.0 for 164 candidate markers that clearly demonstrated inheritance from the F1 parent and no distortion from a 1:1 ratio at $p \leq 0.05$. Under these conditions, a total of 129 markers were linked to each other within the following groups: eleven linkage groups containing a range of 5-17 markers, two groups with 4 markers, and one pair. Individual analysis of additional candidate framework and accessory markers at the same stringencies added 72 markers; this created thirteen linkage groups containing between 7 and 31 markers. The final linkage maps were produced by a total of 201 markers (Figure 3.1, Tables 3.2 and 3.3), and 194 candidate markers were not placed on the map. Linkage groups contained between 7 and 31 markers and ranged in size (cM) from 14.22 to 60.92; the average length was 37.38 cM. The total map length was 485.93 cM, Kosambi.

Again, 201 framework and accessory markers scored across 84 progeny were used to construct the final linkage maps for the F1 parent; these 201 markers represent 32.4% of the total polymorphisms scored. The thirteen linkage groups assembled in JOINMAP® software were analyzed in MAPMAKER™ software for framework map construction. There were 51 framework markers and an additional 150 accessory markers; these figures constituted 8.2% and 24.1% of the total polymorphisms scored, respectively.

Of the 201 mapped markers, 161 markers segregated in a 1:1 pattern at some level, and 40 segregated in a 3:1 pattern at some level. Only 2 framework markers and 18 accessory markers were distorted from their

expected Mendelian ratios at $p \leq 0.05$ but not at $p \leq 0.01$. Obviously, relatively large numbers of accessory markers were generated by the use of markers segregating in a 3:1 ratio in addition to markers which were not significantly different from a 1:1 segregation ratio but which could not be placed as framework markers due to the methods and stringencies employed.

Few maps within the genus *Castanea* have been previously published (Casasoli *et al.* 2001, Casasoli *et al.* 2004, Barreneche *et al.* 2004, Kubisiak *et al.* 1997, Clark *et al.* 2001), and only one study (2 maps) (Casasoli *et al.* 2001) has been published in a format similar to that which was reported in this study and which readily distinguishes between framework and accessory markers. This prior study by Casasoli *et al.*(2001) employed ISSRs, RAPDs, and isozymes to map two *C. sativa* individuals. In those two maps, only 92 (41.1%) markers out of 224 mapped markers and 95 markers out of 175 mapped markers (54.3%) were used as framework markers, respectively, yet these maps provided an estimated 76% and 68% genome coverage. In comparison, 25.3% of the markers mapped in the current study were used as framework markers. This may be due in part to a smaller sample size in the current study and by the exclusive use of 3:1 markers as accessory markers in this study but sometimes as framework markers in the earlier study. Thus, current and prior research show that it is not unreasonable for genomic maps of *Castanea* spp. to demonstrate a relatively high proportion of accessory markers, especially when compared to maps constructed for conifer species (Remington *et al.* 1999).

Due to the method of framework map construction and individual testing and placement of accessory markers, the stringent LOD value, and the interval support where $LOD \geq 3$, it can be assumed that the order of the framework markers was correct and that additional individuals in the mapping population should not change the relative order of the existing framework markers. The order was not likely to change with the addition of more loci or individuals, however it is possible that more framework markers could be added to the map, and that the recombination fractions would decrease with additional information.

3.2.3. Estimates of Genome Size and Coverage and Average Distance between Framework Markers

The total length of the framework map was 486 cM distributed over 13 linkage groups. Genome size was estimated to be L= 621 cM using the method described by Hulbert *et al.* (1988) and Chakravarti *et al.* (1991) with a modified estimator described by Remington *et al.* (1999), where n = 211, d = 24, k = 1314, and C=12. According to this estimate, the linkage map constructed in this study covered 78.13% of the genome.

The average distance between framework markers was 12.79 cM, Kosambi, using the method described by Remington *et al.* (1999), where the map length was 485.93 cM, the number of framework makers was 51, and the number of linkage groups was 13.

Published estimates of genome size, in conjunction with cytological observations, suggest that the species within the genus *Castanea* have fairly small genomic lengths. In Kosambi units, previously published estimates for total genomic lengths vary from 700-721 cM for *C. dentata* x *C. mollissima* F2 hybrids (Kubisiak *et al*. 1997, Clark *et al.* 2001) to 947 - 1,110 cM for *C. sativa* (Cassoli *et al.* 2001; Cassoli *et al.* 2004). Other published research suggests that the chestnut may have an especially short genome length in comparison to conifer species. Numerous published genetic maps of pine species cover between 1170 - 2285 cM, Kosambi, and maps of other conifers species cover up to 3000 cM, Kosambi (Hudson 2005). Karyomorphological studies also support a relatively short genome length of *Castanea* compared to conifer species. Reported chromosome lengths of *C. crenata* ranged from 0.8 – 2.5 µm (Tanaka and Oginuma 1986), while chromosome lengths discussed in a study of eight pine species and eight other conifer species varied between approximately 8.8 – 15.5 µm (Muratova 1994). Thus, reported map distances of chestnuts are up to 4.2 times smaller, and published chromosome lengths have been reported as 6.2 to 11 times smaller in chestnuts than in conifer species.

3.2.4. Marker Synteny

When linkage groups from this study were compared with maps from Clark *et al.* (2001), synteny was found between 24 markers; these markers were shared between 11 groups in the current study and 10 groups in the previous study. Each association between maps was formed on the basis of 1 to 4 similar markers. (Table 3.3) The linkage groups in the previous study were labeled as framework groups A-O (no M) and framework marker pairs 1 and 2, while the linkage groups in the current study were labeled 1-13. Each linkage group from the current study, except for groups 6 and 10, could be associated with one of the prior linkage groups by 1-4 markers.

In all cases, except between groups 7 and B and between groups 8 and H, the order of shared markers was maintained between maps. In some instances, however, this observation was less reliable due to differences in presentation style between the maps—the current study differentiates between framework and accessory markers, while the previous study does not.

There was only one instance where markers from a linkage group in the current study showed synteny with two groups from previous research. In this case, marker markers ACA/CTA_131.3X and AGC/CAC_170.2x* showed synteny with markers e35m59-131.2 in group K and e40m48-169.0 in group C, respectively. There was also one case where markers from two different groups in the current study showed synteny with a single group from the previous study. In this case, three markers from group 4 and a single marker, ACG/CTT_192.6Z, from group 3 were all associated with group B/E. Given the length of groups 3 and 4 (54.7 and 54.1, respectively), it is unlikely that these groups are located on the same chromosome. No other contradictory cases of marker synteny were found.

Post-mapping SSR analysis of the population studied by Kubisiak *et al.* (1997) has indicated that 18 individuals (17.6%) were found to be the result of pollen contamination and that corrections needed to be made to the map generated by that study, although exactly how these contaminating individuals may have affected the later map generated by Clark *et al.* (2001) was not reported (Sisco *et al.* 2005). It would not be

unreasonable to assume that contaminating individuals may have resulted in misplaced markers in the previous map, which could lead to the contradictory cases of synteny described above. While it is possible that contaminating individuals exist within the samples used in the current map, a visual examination of the gels during the scoring process did not generate concern regarding individuals with unusual marker patterns.

It is interesting to note that three markers show synteny between linkage groups 11 and B and that two markers show synteny between groups 7 and F. These linkage groups are the site of the 2 major loci governing resistance to *Cryphonectria parasitica*, (Murrill) Barr (Kubisiak *et al.* 1997; Sisco *et al.* 2005). In particular, locus $X03_{1050}$ in group B is bounded on one side with three markers shared between maps; these markers are AGC/CAC-277.4, ACA/CGT-149.2, and AGC/CAC-139.5 and are located within 0.7, 2.9, and 13.7 cM, Kosambi of the resistance locus, respectively. The other locus governing resistance, $B03_{1675}$ in group F, is located within 10.4 cM of marker ACA/CGT-256.2 on one side and within 12.7 cM on the other side by marker ACA/CCG-228.9X.

3.2.5. Marker Origin from the 'Mahogany' Grandparent

Mapped markers with a band present in the *C. mollissima* 'Mahogany' grandparent are listed Table 3.4; genotype scores for both parents are also included. Bands which appear in the 'Mahogany' genotype but are absent from WB348 genotype would indicate primer binding sites that are derived from the 'Mahogany' grandparent, and which might possibly indicate genomic regions that are present in *C. mollissima* but absent from *C. dentata.* These markers might prove useful in future efforts to map traits inherited from the 'Mahogany' grandparent.

3.3. Future Work

The map from this study is the first known map composed entirely of AFLP markers for a *C. mollissima* x *C. dentata* individual. It would be helpful to identify additional framework markers to strengthen the map and provide increased coverage. In a genome of this size, an increased progeny set may alleviate some of

the difficulty in achieving the precision required to produce a more informative map. A more rigorous search for contaminating individuals through the use of codominant markers, such as microsatellites, may also improve the quality of the linkage maps produced from the data set in this study.

If reliable phenotypic *P. cinnamomi* resistance data can be obtained for this population, it may be possible to use this map to determine the number and position of loci which would confer disease resistance. During the summer of 2006, surviving specimens from the 2005 *P. cinnamomi* root inoculation trials were repotted in larger containers with the same type of medium, placed in the outdoor facilities used in 2003 and 2004, and will be re-inoculated during the spring of 2007. Hopefully, this testing will provide a clear segregation pattern. A 1:1 ratio of segregation for resistance to disease is hypothesized for family KY115xWB348, based on Mendelian segregation patterns for a first-generation backcross population. If this hypothesis is supported by the segregation data, it may be possible to map a single locus controlling resistance using the genetic map developed from AFLP markers described in this chapter.

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Table 3.1 Primers used for selective amplification in this study. For 12 selected primers, the number of fragments scored and the number of markers mapped in this study are compared to the number of markers mapped by Clark *et al.* (2001) using the same primers. Segregation distortion information is also provided for markers from the current study.

Primer	Number of Fragments Bowles	Number of Mapped Markers Clark	Number of Mapped Markers Bowles	Number of Markers Without Linkage Distortion – Bowles			
				1:1 $p < 0.05$	1:1 $p < 0.01$	3:1 $p < 0.05$	3:1 $p < 0.01$
ACA/CCA	23	11	5	3	2		
ACA/CCC	23	9	3	2		1	
ACA/CCG	15	11	5	4	1		
ACA/CCTA	14	-	3	1	1		1
ACA/CGT	17	12	8	6	1	1	
ACA/CTA	25	20	13	10		3	
ACG/CGC	18	15	-				
ACG/CTA	13	9	7	4		2	1
ACG/CTT	29	19	8	8			
AGC/CAC	25	13	9	7		2	
AGC/CCA	22	16	-				
AGC/CTC	25	15	8	5		1	2
AGG/CCA	14	-	2	2			
AGG/CCGC	9	-	3	3			
AGG/CCG	13	-	2	2			
AGG/CCTA	17	-	11	10		1	
ATG/CAC	25	-	9	6	1	2	
ATG/CCAG	8	-	3	2		1	
ATG/CCCA	22	-	9	8		1	
ATG/CCG	18	-	2	1			1
ATG/CCTA	25	-	12	8	1	3	
ATG/CTA	46	-	23	20		2	1
ATG/CTC	12	-	5	3		1	1
ATG/CTG	28	-	12	7		5	
ATT/CAC	14	-	4	4			
ATT/CCGG	14	-	4	3		1	
ATT/CCTA	8	-	3	2		1	
ATT/CGC	16	-	3	3			
ATT/CTA	27	-	16	10	3	2	1
ATT/CTC	14	-	2	1			1
ATT/CTG	23	26	4	3		1	
ATT/CTT	19	-	3	2	1		

Table 3.2 Accessory markers assigned to their respective framework marker by linkage group (LG).

Linkage Group	Framework Marker	Accessory Marker
1	ATG/CTA_519.0X	-
	ACG/CTT_690.6Y	AGG/CCGG_132.6X ATT/CTA_336.6X† AGG/CCGG_316.7Y
	AGC/CTC_90.2X	ATG/CTC_148.0Y* ATT/CTG_188.8X* ACA/CCTA_75.7Y* ATG/CCAG_255.7Y ATG/CCCA_162.7x
	AGC/CTC_239.8Z	-
	ATT/CTG_261.3X	-
	AGG/CCGC_79.5Y	-
2	AGG/CCTA_79.2Z	-
	ATG/CTG_513.8Y	
	ATG/CTG_473.3Y	ACA/CCC_387.9Y
	ATG/CTG_285.3Y	ACA/CTA_616.1Z ATG/CCCA_112.2X ATG/CAC_265.5Y
	ATG/CAC_415.2Y	-
	AGC/CTC_179.6x	AGC/CAC_170.2x* ACA/CTA_131.3X ATG/CTA_73.3Y ACA/CCG_101.7Z† ATT/CTT_100.7X ATT/CTT_400.3Y ACA/CGT_155.5Y ATG/CTG_144y ATG/CTC_119.7y ACA/CCG_156.6x AGC/CTC_365.6z ATG/CCTA_61.3z†
	ATT/CCGG_350.4y	-

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.2 Continued

Linkage Group	Framework Marker	Accessory Marker
3	AGC/CAC_208.7Y	-
	ATG/CTA_562.8Z	ATG/CCTA_466.5X ATG/CCTA_93.8X
	ATG/CTA_477.8Z	AGG/CCTA_144.8X
	ATT/CCTA_345.0X	ATT/CTA_545.9Y ACG/CTT_192.6Z ACA/CCA_378.5Y†
	ATT/CTA_268.5x	ATT/CTA_193.6Y
4	AGC/CAC_729.0Z	ATT/CAC_102.0.1Z
	ACG/CTT_655.6Z	ATG/CTA_389.7Z* ACG/CTA_179.4Z* ACG/CTT_358.5X ATG/CTA_323.2X ATT/CTC_272.2Y* AGC/CTC_274.0Y* ATG/CTC_76.5Z* ACA/CGT_269.0Y
	ATG/CTG_446.7y	ATG/CTA_122.2z ATT/CTA_498.2Y† ATG/CCCA_651.2Y ACA/CCTA_349.3y† ACG/CTT_180.6X AGG/CCTA_159.6x ATG/CCTA_252.4Y
	ATG/CTA_110.1z	ACA/CTA_117.7X* ATG/CAC_227.8Y* AGC/CTC_101.1X* ATG/CCG_146.9Z ATG/CTC_172.7Z
	ATG/CCAG_211.8Z	ATG/CCAG_213.6Z*

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.2 Continued

Linkage Group	Framework Marker	Accessory Marker
5	ATT/CCTA_245.9Y	ACA/CTA_220.2y
		ATG/CTA_279.7z
		ATG/CTA_578.5Y
		ATT/CTA_327.7Y
		ATG/CCTA_491.3Z*
		ATG/CCGG_238.9Y*
		ATG/CTA_282.7Z
		ATG/CTA_586.0Y
		ATG/CTA_610.7Z
		ATT/CTA_482.5Y
		ACG.CTA_150.9Z*
		ACA/CTA_148.8X*
		ATG/CCCA_107.0Y*
	ATT/CTA_219.4Y	ACG/CAC_379.8Z
		AGG/CCGC_129.2Z
		ACG/CTT_408.4Y
		AGC/CAC_385.2Z*
		ACG/CTA_116.8Z*
		ATG/CAC_85.4Y*
		ATG/CTA_263.1X*
		ATT/CCTA_351.5Z*
		ATT/CTA_133.9Y
		ATG/CAC_167.0Z*
		ATG/CTG_184.0X*
		ACA/CCC_118.8Y
		ATG/CTG_165.7Y*
	ATG/CAC_191.3y	ATG/CAC_167.0Z*
	ACA/CCA_208.7X	-
6	ACG/CTA_320z	-
	ACG/CTA_317.4y	ATG/CTA_329.3z
		ATG/CTA_303.0Y
		ATG/CTA_245.3Y
		ATG/CTG_111.5Z
	AGG/CCAG_88.3Z	AGG/CCTA_88.4Z
		ACA/CCG_312.2Y
		AGC/CAC_139.0X
		ACA/CGT_149.0X
		ATG/CTA_219.3z
		ATG/CCTA_123.3Y
		ACA/CCG_110.8Z
		AGC/CAC_277.8Y
		ACA/CTA_385.9X
		ATT/CTA_332.8Y

* indicates markers segregating in a 3:1 ratio
† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.2 Continued

Linkage Group	Framework Marker	Accessory Marker
7	AGG/CCTA_216.2Y	ATG/CTA_138.9Z* ACA/CTA_114.2X*
	ATG/CCTA_575.7Y	ACA/CCA_151.8Y† ATG/CTA_230.8Y ATG/CTA_441.3Z ATG/CTA_137.1z AGC/CAC_197.2x
	ATT/CGC_211.7Y	ACA/CCA_103.9x
	ATT/CAC_196.1Y	-
	ACA/CGT_142.8X	ACA/CTA_160.0X ATG/CTA_594.4Z ATG/CCTA_276.8Y* ACA/CTA_351.5X ATT/CTA_199.3Y†
8	ACA/CTA_389.0X	-
	ATT/CTA_388.6X	ATG/CTG_459.8Y ATG/CTA_169.3Y ATG/CCTA_130.4Z ATT/CTA_422.4Y ACG/CTA_239.0Z ATG/CCTA_279.5Y
	ATT/CCGG_159.5Y	-
9	ACA/CGT_359.3X	ATG/CTC_134.9X ATG/CAC_397.8Z
	ATT/CGC_466.8Z	ACA/CGT_107.1X ATT/CAC_163.6Y ACG/CTA_439.5Y ACA/CCA_308.4Y† ACA/CTA_440.1Z ATG/CCCA_224.0X
	AGG/CCTA_340.4Z	AGC/CAC_142.0Y AGG/CCAG_338.8Z

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.2 Continued

Linkage Group	Framework Marker	Accessory Marker
10	ACA/CGT_254.2	ATG/CCTA_ 430.2Z
	ATT/CTC_69.3Y	ATG/CTA_227.6Y
	ATG/CCTA_171.3X	AGG/CCTA_92.6X ATG/CCCA_430.8X
11	ACA/CGT_256.7X†	ACA/CCG_271.1y
	ATG/CAC_280.3Y†	ATG/CTG_186.4X
	ATG/CTA_284.7Z	ACG/CTT_74.4X ATT/CTA_246.0z* ACA/CTA_560.3z ACG/CTT_518.0z
12	ACG/CTT_142.8X	ATG/CTG_297.5y* ATG/CTG_163.5x* ACA/CGT_240.3x*
	AGG/CCTA_476.8y	ATT/CGC_65.6Z ATT/CCGG_151.6Y AGG/CCTA_205.8Z AGC/CTC_203.1z
13	ATG/CAC_126.9Y	AGC/CTC_116.7X* ATG/CCCA_168.2Y ATG/CTG_499.4y* ATG/CCG_501.4Y*† ATT/CTC_267.0z*† ATG/CCTA_261.2y*
	ATT/CTG_242.5X	AGG/CCGC_148.0Y ATT/CAC_175.0Y ATT/CTG_651.8Z ACA/CCC_410.5Y*

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.3 Markers showing synteny between the genetic linkage map in the current study and Clark *et al.* (2001).

Clark		Bowles	
Linkage group	Loci	Linkage Group	Loci
A	e35m59-221.5	5	ACA/CTA_220.2y
	e35m59-150.6	5	ACA/CTA_148.8X*
	e35m52-118.9	5	ACA/CCC_118.8Y
	e37m59-118.1	5	ACG/CTA_116.8Z*
B	e40m48-277.4	7	AGC/CAC_277.8Y
	e35m58-149.2	7	ACA/CGT_149.0X
	e40m48-139.5	7	AGC/CAC_139.0X
B/E	e40m60-101.6	4	AGC/CTC_101.1X*†
	e37m62-191.3	3	ACG/CTT_192.6Z
	e40m60-273.0	4	AGC/CTC_274.0Y*†
	e37m59-180.9	4	ACG/CTA_179.4Z*†
C	e40m48-169.0	2	AGC/CAC_170.2x*
	e40m60-117.5	13	AGC/CTC_116.7X*
	e46m61-243.7	13	ATT/CTG_242.5X
D	e40m60-241.3	1	AGC/CTC_239.8Z
	e46.61-263.1	1	ATT/CTG_261.3X
F	e37m65-228.9	11	ACA/CCG_227.1y
	e35m58-256.2	11	ACA/CGT_256.7X†
G	e35m51-308.7	9	ACA/CCA_308.4Y†
H	e37m59-240.3	8	ACG/CTA_239.0Z
	e35m59-389.5	8	ACA/CTA_389.0X
J	e35m58-240.7	12	ACA/CGT_240.3x*
	e37m62-143.7	12	ACG/CTT_142.8X
K	e35m59-131.2	2	ACA/CTA_131.3X

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.4 Markers that were mapped in the current study and were band present in *Castanea mollissima* 'Mahogany' grandparent genotype.

mapped name	'Mahogany'	F1	American
ACA/CCA_151.8†	1	1	2
ACA/CCA_208.7X	1	1	2
ACA/CCA_308.4Y†	1	1	2
ACA/CCC_387.9Y	1	1	2
ACA/CCC_410.5*	1	1	1
ACA/CCG_312.0Y	1	1	2
ACA/CGT_142.8X	1	1	0
ACA/CGT_149X	1	1	2
ACA/CGT_256.7X†	1	1	2
ACA/CGT_359.3X	1	1	2
ACA/CTA_117.7X*	1	1	0
ACA/CTA_148.8X*	1	1	2
ACA/CTA_160.0X	1	1	2
ACA/CTA_245.3Y	1	1	2
ACA/CTA_351.5X	1	1	2
ACA/CTA_389.0X	1	1	2
ACA/CTA_440.1Z	1	1	2
ACA/CTA_616.1Z	1	1	2
ACG/CTA_150.9Z*	1	1	2
ACG/CTA_179.4Z*†	1	0	2
ACG/CTA_239.0Z	1	1	2
ACG/CTA_439.5Y	1	1	2
ACG/CTT_142.8X	1	1	2
ACG/CTT_180.6x	1	1	2
ACG/CTT_358.5X	1	1	2
ACG/CTT_408.4Y	1	0	2
ACG/CTT_74.4	1	1	2
AGC/CAC_139X	1	1	0
AGC/CAC_208.7Y	1	0	2
AGC/CAC_277.8Y	1	1	2
AGC/CAC_379.8Z	1	1	2
AGC/CAC_385.2Z*	1	1	2
AGC/CTC_116.7X*	1	1	2
AGC/CTC_239.8Z	1	1	2

* indicates markers segregating in a 3:1 ratio
† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.4 (cont.)

mapped name	'Mahogany'	F1	American
AGC/CTC_90.2X	1	1	2
AGG/CAC_102.1Z	1	1	2
AGG/CCAG_338.8Z	1	1	2
AGG/CCAG_88.3z	1	1	2
AGG/CCCG_316.7Y	1	1	2
AGG/CCGC_129.2Z	1	1	2
AGG/CCGC_79.5Y	1	1	2
AGG/CCTA_144.8	1	1	2
AGG/CCTA_205.8Z	1	1	2
AGG/CCTA_340.4Z	1	1	2
AGG/CCTA_92.6X	1	1	2
ATG/CAC_126.9Y	1	1	2
ATG/CAC_167.0Z*	1	1	2
ATG/CAC_265.5Y	1	1	2
ATG/CAC_280.3Y†	1	1	2
ATG/CAC_397.8Z	1	1	2
ATG/CAC_415.2Y	1	1	2
ATG/CAC_85.4Y*	1	1	2
ATG/CCAG_211.8Z	1	1	2
ATG/CCAG_213.6Z*	1	0	0
ATG/CCCA_107.0Y*	1	1	2
ATG/CCCA_112.2X	1	1	2
ATG/CCCA_130.4Z	1	1	2
ATG/CCCA_168.2Y	1	1	2
ATG/CCCA_224.0X	1	1	2
ATG/CCCA_651.2Y	1	1	2
ATG/CCG_146.9Z	1	1	2
ATG/CCG_501.4y*†	1	1	1
ATG/CCTA_123.3Y	1	1	2
ATG/CCTA_171.3X	1	1	2
ATG/CCTA_279.5Y	1	1	2
ATG/CCTA_349.3†	1	1	2
ATG/CCTA_466.5X	1	1	2
ATG/CCTA_491.3Z*	1	1	2
ATG/CCTA_575.7Y	1	1	2
ATG/CCTA_93.8X	1	1	2
ATG/CTA_156.1Y	1	1	2
ATG/CTA_169.3Y	1	1	2

* indicates markers segregating in a 3:1 ratio
† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

Table 3.4 (cont.)

mapped name	'Mahogany'	F1	American
ATG/CTA_279.7Z	1	1	2
ATG/CTA_282.7Z	1	1	2
ATG/CTA_284.7Z	1	1	2
ATG/CTA_477.8Z	1	1	2
ATG/CTA_519.0X	1	1	2
ATG/CTA_562.8Z	1	1	2
ATG/CTA_594.4Z	1	0	0
ATG/CTA_610.7Z	1	1	2
ATG/CTC_134.9X	1	1	2
ATG/CTC_148.0Y*	1	1	1
ATG/CTC_172.7Z	1	1	2
ATG/CTG_111.5Z	1	1	2
ATG/CTG_186.4X	1	1	2
ATG/CTG_194.8Y	1	1	2
ATG/CTG_285.3Y	1	1	2
ATG/CTG_459.8Y	1	1	2
ATG/CTG_473.3Y	1	1	2
ATG-CCG_230.4y*	1	1	2
ATT/CAC_163.6Y	1	1	2
ATT/CAC_196.1Y	1	1	0
ATT/CCCA_64.3x*	1	1	2
ATT/CCGG_151.6Y	1	1	2
ATT/CCGG_159.5Y	1	1	2
ATT/CCGG_238.9Y*	1	1	2
ATT/CCTA_351.5Z*	1	1	0
ATT/CGC_466.8Z	1	1	2
ATT/CGC_65.6Z	1	1	2
ATT/CTA_193.6Y	1	1	2
ATT/CTA_219.4Y	1	1	2
ATT/CTA_388.6X	1	1	2
ATT/CTA_422.4Y	1	1	2
ATT/CTA_482.5Y	1	1	2
ATT/CTC_267.0z*†	1	1	1
ATT/CTG_188.8X*	1	1	1
ATT/CTG_242.5X	1	1	2
ATT/CTG_261.3X	1	1	2
ATT/CTG_651.8Z	1	1	2

* indicates markers segregating in a 3:1 ratio

† indicates markers that are distorted at $p \leq 0.05$, but not at $p \leq 0.01$

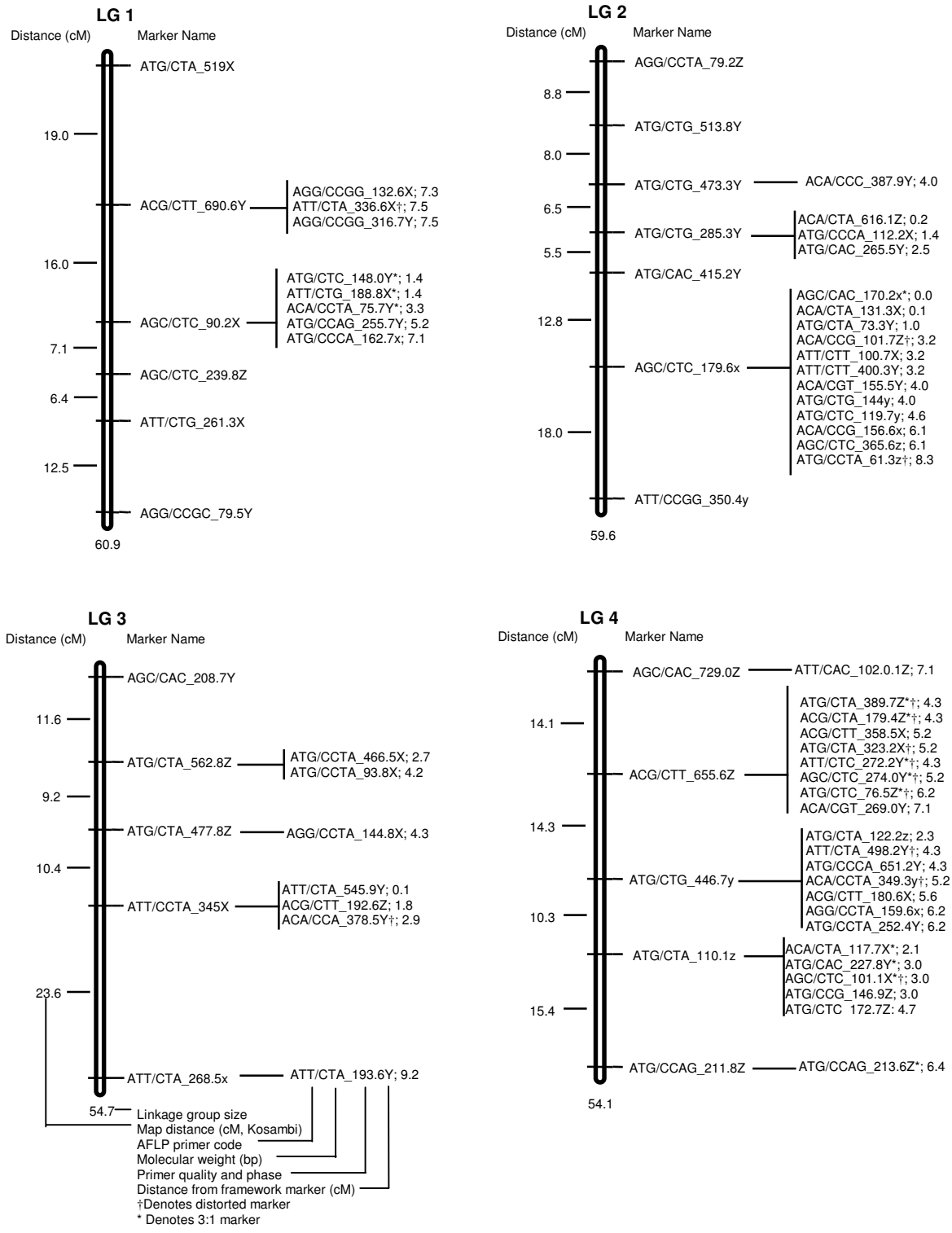

Figure 3.1 Genetic linkage map of 'KY115' (*Castanea mollissima* 'Mahogany' x *Castanea dentata* 'RCF1'). The map is 486 cM, Kosambi, scored from 30 primer pairs. 13 framework groups were constructed at LOD ≥ 5.0 and θ ≤ 0.40. The framework map was constructed using 52 markers: 50 undistorted 1:1 markers at $p \leq 0.05$, and 2 1:1 markers that were undistorted at $p \leq 0.01$. Interval support was ≥ 3.0. The 150 accessory markers were placed on the framework map using MAPPOP or MAPMAKER software. * indicates 3:1 markers; † indicates markers distorted at $p \leq 0.05$, but not at $p \leq 0.01$.

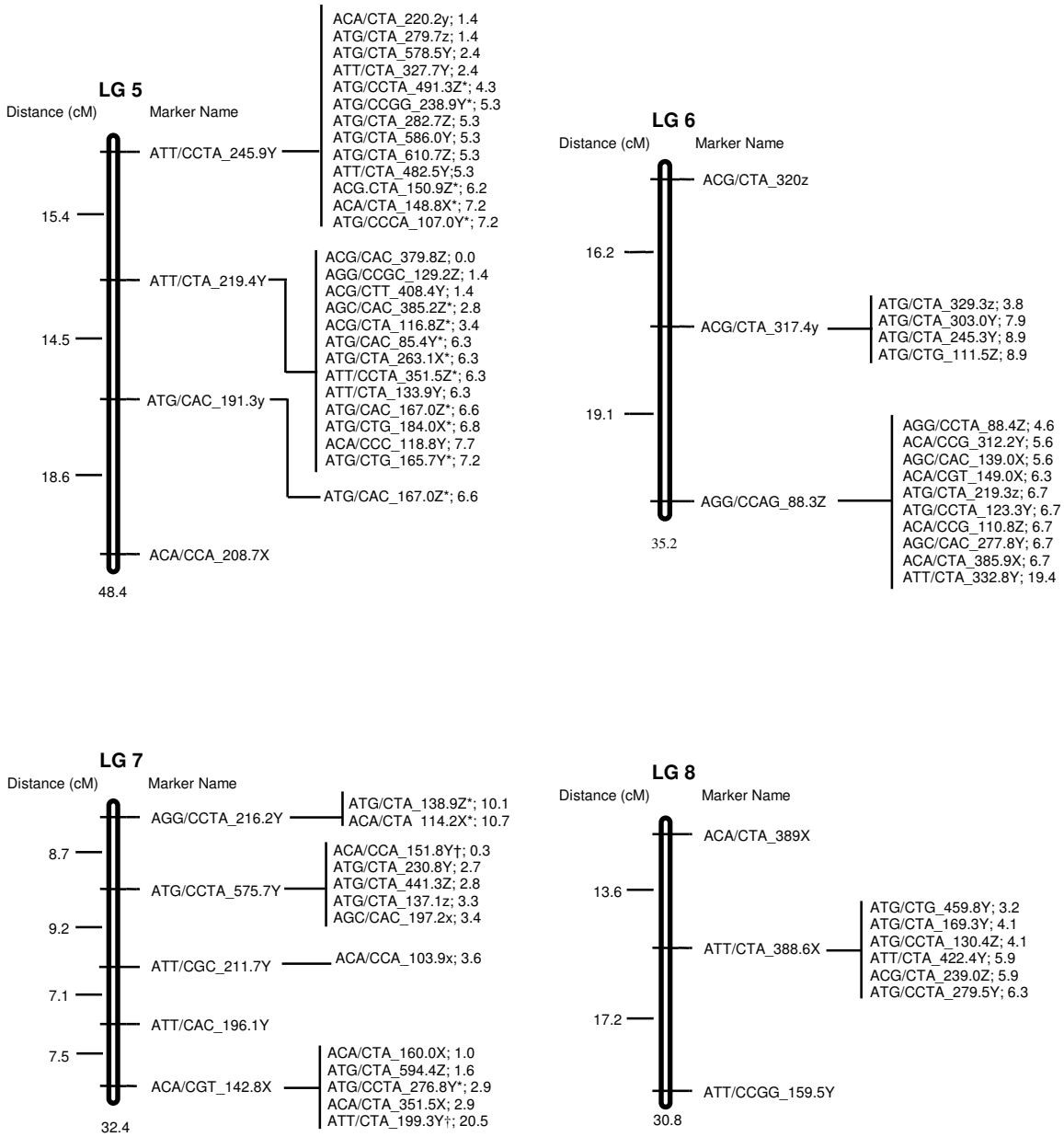

Figure 3.1 Continued

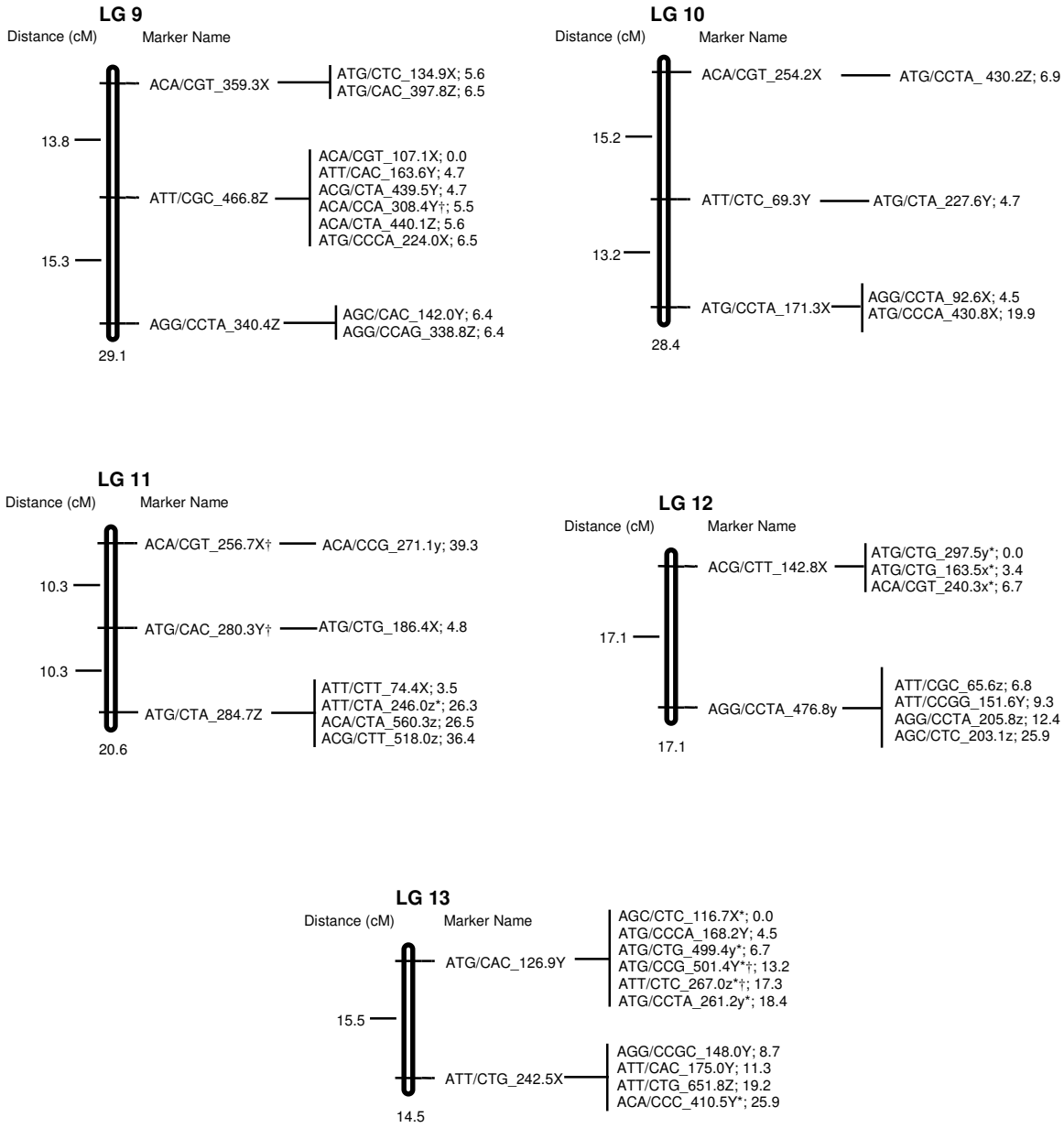

Figure 3.1 Continued

APPENDIX

SAS Code Used for AFLP Marker Analysis

This code is designed to process diploid AFLP scores based on the genotypes of a B1 progeny population, both parents, and one grandparent (Chapter 3). This program has several functions, which were useful in analyzing the segregation pattern, quality, and parental origin of each polymorphism. The program starts by converting QUANTAR data to a useable format, and produces data sets that can be used in JOINMAP and MAPMAKER software, respectively.

To make use of the data conversion step from QUANTAR, the user needs to compile the QUANTAR data in a spreadsheet format, aligning all scored polymorphisms by sample and providing marker names in the format required for MAPMAKER. The program will delete all polymorphisms that contain ≥20% unknown scores, but it cannot delete progeny samples (individual specimens, which are in columns) based on a percentage of unknown scores for each sample. (Thus, if samples are to be deleted from the data set, the data must be exported to a spreadsheet, examined manually, and then be re-imported.)

The program's next function is to determine the segregation pattern for each polymorphism based on 'band–present' frequency. This is done using a chi-square test at the $p \leq 0.05$ and $p \leq 0.01$ levels, where the null hypothesis is that the polymorphism is not significantly different from a 1:1 ratio. Because this particular data set is derived from diploid DNA, another test is run (at the same confidence levels) where the null hypothesis is that the polymorphism is not significantly different from a 3:1 ratio.

The parental origin of each polymorphism is then derived based on the parental genotype scores. The data is then presented in a list format sorted by primer combination. This list includes a yes/no column, which suggests whether each polymorphism should be re-scored. Markers that are listed as 'yes' include cases that are significantly distorted from a 1:1 or a 3:1 pattern and cases where the segregation pattern and the parental origin do not agree (for example, 1:1 polymorphisms where both parental genotypes are unknown, or cases where the polymorphism segregates in a 3:1 ratio, but only 1 parent has a band present). Other

output produced at this point includes a list and a summary table of the polymorphisms according to their segregation pattern (1:1 at $p \leq 0.05$, 1:1 at $p \leq 0.01$, *etc*.). It is assumed that the user would rescore the polymorphisms as desired, and import the new data set into the program. The program can be run from the beginning again.

Suffixes are created for each polymorphism that a) does not fall into a segregation pattern of 1:1 at $p \leq 0.05$ *and* b) is not from the F1 parent of interest. These suffixes describe the segregation pattern for each band, and whether the scores for at least one of the parents are unknown. These suffixes are carried through to the end of the program and are attached to the name of each polymorphism in the output data sets so that this information is known for each marker throughout map construction. The meaning of each suffix is fairly straightforward, but it is also listed in the SAS code.

All polymorphisms are then deleted which a) are distorted from a 1:1 or 3:1 ratio at $p \leq 0.01$, *or* b) are not distorted from a 1:1 pattern, but do include a band-present in the American parent (the parent that is not of interest). This leaves a data set containing polymorphisms that a) segregate in a 1:1 pattern at either confidence level and are known to be inherited from the F1 parent of interest; *or* b) segregate in a 1:1 pattern at either confidence level and have an unknown parental origin (except for cases where the American parent has a band-present); *or* c) segregate 3:1 at either confidence level, regardless of available parental genotypes.

The program's next functions are to format and export the remaining data set in two formats. The first format is for JOINMAP according to the CP mating design as described in the JOINMAP® manual, tables 5-7, pp. 37 & 40. Polymorphisms segregating 1:1 with an unknown parental origin are coded once as being inherited from the F1 parent, and coded in duplicate as being inherited from the American parent. Polymorphisms that are not significantly different from both a 1:1 *and* a 3:1 pattern *and* that could not be assigned to a segregation pattern during previous steps based on parental origin are a special case; these bands are coded as 3:1 markers, and given their own suffix (again, refer to code and output). A duplicate data set is produced in a second format that is coded for use in MAPMAKER. For this data set, bands

segregating 3:1 are coded so that the band-present scores are listed as unknown and only the band-absent scores are reported. Before it can be used however, this MAPMAKER data set will have to be duplicated in spreadsheet software in the opposite phase according to requirements of using the haploid data type.

The program's final function is to produce a set of tables summarizing the number of polymorphisms that fall within each segregation pattern, and how many bands within those categories still need rescoring in the parental genotypes. These tables are quite useful.

Again, the code in the following SAS software program was designed for use with the particular data set used in this study and described in Chapter 3. Editing would therefore be required before this code could be applied to another data set. Obvious changes would include altering the number of progeny samples, the names of the parents, and source and output filenames. The user should be careful to read through the entire program, making all changes as necessary. While this program is very specific to this data set, it should serve as a useful model for sorting out polymorphisms produced in other projects which employ diploid DNA samples.

SAS Code Used for AFLP Marker Analysis

```
*********************************************************************
*********************************************************************
***                                                               ***
***   SAS PROGRAM TO PREPARE DIPLOID AFLP DATA FOR MAPPING        ***
***                                                               ***
***                      Mollie E. Bowles                         ***
***                      Dr. John Frampton                        ***
***                         CTG, NCSU                             ***
***                       June 19, 2006                           ***
***                                                               ***
***               adapted from a program written                  ***
***         by Dr. Frampton to process haploid data for           ***
***         Emily Hudson's (CTG, NCSU) Master's Thesis            ***
***                                                               ***
***                      INSTRUCTIONS BELOW!!!                    ***
*********************************************************************
*********************************************************************

************    Input to use for this SAS program    ************

1.  Build a file called "chXX-original"
      *This file should be the
            *FULL data set as output from Quantar
            *WITH TREE NAMES in the first row
            *with CORRECTLY FORMATTED MARKER *NAMES*
            *where samples and markers are SORTED and ALIGNED by id #

2.  Copy the data set from "ChXX-original"
      a. paste it into a new file and save as "chXX-Nameless"
      *this will prevent problematic rows/columns of empty data in SAS
      b. delete the whole first row of tree names and save again
      *this will allow SAS to use the proper column names in the
       default program code

************          RUN THIS PROGRAM             ************

1.    READ the program below
      a.    CHANGE any source filenames
      b.    ADD/DELETE/CHANGE SAS number of samples, columns names, and
            parent/grandparent names as needed

2.    RUN the program
      The program will:
      1. Convert the Quantar-formatted data to SAS format
      2. Delete all markers with zero-score frequencies greater than or
         equal to 20%
      3. Separate the components of the maker name for easier sorting,
         reporting, and analyzing markers
      4. Determine segregation patterns based on marker frequency at
         p=.05 and p=0.1
      5. Determine the marker origin based on parental data at p=.05
      6. Produce tables and lists detailing the results of #4 and #5
         for each marker
```

```
        *Print a summary table of the number of markers at each
         segregation distortion level
        *Print markers in list form by segregation classification,
         by band-present frequency
        *Print markers in list form indicating which markers may need
         to be rescored because of a lack of information or an
         unpredicted conflict between the presence of the marker in the
         parent genotype and the segregation pattern at p < 0.05 or
         p < 0.01
      7. Create marker suffixes for each marker's segregation pattern
         and parental origin
      8. Produce, format, and export a single data set suitable for use
         in Mapmaker containing only the following marker types:
         *markers that segregate 1:1 or distorted 1:1 and are of
          unknown origin in both parents
         *markers that segregate 1:1 or distorted 1:1 and are
          present in the F1 parent
         *markers that segregate 3:1 and are present in both parents
         *markers that segregate 3:1 and are of unknown origin in one
          or both parents
         *(the program deletes
            all distorted 1:1 markers
            all markers that segregate in a 1:1 pattern AND are present
            in the American parent)
       9. Format & export the new data for Joinmap according to the CP
          mating design as described in the Joinmap® 3.0 manual,
          tables 5-7, pp.37&40

   *Note* Data from the exported files (steps 9 and 10) are in a format
          suitable for deleting samples with high 0-scores (in Excel)
          and then processing in the preferred program
   To do this:
      1. Paste in the sample id's from "chXX-original" into
         "chXX-SASoutput"
         (this permits identification of the samples deleted or
          retained)
         (save the 'named' file as "chXX-SASnamed")
      2. Calculate the percent 0-scores for the samples
         (save this file as "chXX-SASpercents")
      3. Delete samples (columns) with > 20% 0-scores in excel
         (save this file as "chXX-SASdeleted")
      4. Save the file for mapping use, as either "chXX-Joinmap" or
         "chXX-Mapmaker";
*********************************************************************
*********************************************************************;

Options PageNo=1;
Title1 'Mollie''s AFLP Markers: CH42';
Title2 'All  Primer Combinations';


                *** 1. Formatting QUANTAR data into SAS format ***;;

Proc Import datafile='C:\Thesis\ch42-less9s.xls'
OUT=Sasuser.ch42less9s replace;
getnames=no;
```

```
run;;

Data Mollie01; Set SasUser.ch42less9s;
Array F(91);
Array G(91);
      Do I = 2 to 91;
      If F(I) = '+' then G(I) = 1; *marker present;
        If F(I) = '-' then G(I) = 0; *       absent;
        If F(I) = '?' then G(I) = .; *       unknown;
        If F(I) = 'X' then G(I) = .; *sample failure;
   END;
Marker = F1;
IF G89 = 1 then GR = 100; Else GR = G89;
IF G90 = 1 then F1x = 100; Else F1x = G90;
IF G91 = 1 then AM = 100; Else AM = G91;
Drop F1-F91;
Drop I G1;


      *** 2. Drop all markers with 0-scores > 20% (for 88 samples) ***;


zeros = NMISS(of G2-G88);
If zeros GE 88*.2 then DELETE;
drop zeros;


                  *** 3. Substring Primer/MW/Quality ***;;


   Check = Substr(Marker,9,1);
   If Check = '_' then Do;
      Primer  = Substr(Marker,2,7);
        If Substr(Marker,13,1) = '.' then DO;
           xMW = Substr(Marker,10,5);
             Quality = Substr(Marker,15,1);
         End;
         Else Do;
            xMW = Substr(Marker,10,4);
              Quality = Substr(Marker,14,1);
        End;
   End;
   If Check NE '_' then Do;
      Primer  = Substr(Marker,2,8);
        If Substr(Marker,14,1) = '.' then DO;
           xMW = Substr(Marker,11,5);
             Quality = Substr(Marker,16,1);
         End;
         Else Do;
            xMW = Substr(Marker,11,4);
              Quality = Substr(Marker,15,1);
        End;
       End;
   MW = xMW + 0; drop xMW;
run;
```

```
            *** 4a. Segregation patterns at the .05 level ***;;


Data Mollie02; Set Mollie01;
Array G(91);
      Plus = 0; Neg = 0; Num = 0;
      Do I = 2 to 88;
       If G(I) NE . then Do;
            Plus = Plus + G(I);
            Num + 1;
      End;
End;
Neg = Num - Plus;
df = 1;
run;

      *** Calculate Chi-Square Statistics at p=.05***;
Data Mollie03; set Mollie02;
   *** 1:1 Segregation Pattern ***;
   ChiSq11  = ((Plus-Neg)**2)/Num;
   Prob11   = 1-ProbChi(ChiSq11,df);
   IF Prob11 LE 0.05 then Sign11 = 'Yes'; Else Sign11 = 'No';
   *** 3:1 Segregation Pattern ***;
   ChiSq31 = ((Plus-Num*0.75)**2)/Num;
   Prob31   = 1-ProbChi(ChiSq31,df);
   If Prob31 LE 0.05 then Sign31 = 'Yes'; Else Sign31 = 'No';
   ************************************;
   Frequency = Plus/Num*100;
   Test = F1x + AM;
   Rescore = 'No ';
run;


            *** 4b. Segregation Patterns at p=.01 ***;;


data Mollie04; Set Mollie03;
   *** 1:1 Segregation Pattern ***;
   ChiSq1101  = ((Plus-Neg)**2)/Num;
   Prob1101   = 1-ProbChi(ChiSq1101,df);
   IF Prob1101 LE 0.01 then Sign1101 = 'Yes'; Else Sign1101 = 'No';
   *** 3:1 Segregation Pattern ***;
   ChiSq3101  = ((Plus-Num*0.75)**2)/Num;
   Prob3101   = 1-ProbChi(ChiSq3101,df);
   If Prob3101 LE 0.01 then Sign3101 = 'Yes'; Else Sign3101 = 'No';
   ************************************;

   If Sign1101 = 'Yes' and Frequency LT 50
      then Class01 = 'Distorted 1:1         ';
   If Sign1101 = 'No'  and Sign3101 = 'Yes'
      then Class01 = '1:1 Segregation       ';
   If Sign1101 = 'Yes' and Sign3101 = 'No'
      then Class01 = '3:1 Segregation       ';
   If Sign1101 = 'No'  and Sign3101 = 'No' then Do;
      If Test = 200 then Class01 = '3:1 Segregation       ';
```

```
        If Test = 100 then Class01 = '1:1 Segregation       ';
        If Test = 0 or Test = . then Class01 = '1:1 or 3:1
Segregation';
   End;
run;


   *** 5. Segregation Patterns based on Marker Frequency at p=.05 ***;


Data Mollie05; set Mollie04;
   If Sign11 = 'Yes' and Frequency LT 50
      then Class = 'Distorted 1:1         ';
   If Sign11 = 'No'  and Sign31 = 'Yes'
      then Class = '1:1 Segregation       ';
   If Sign11 = 'Yes' and Sign31 = 'No'
      then Class = '3:1 Segregation       ';
   If Sign11 = 'No'  and Sign31 = 'No' then Do;
      If Test = 200 then Class = '3:1 Segregation       ';
      If Test = 100 then Class = '1:1 Segregation       ';
      If Test = 0 or Test = . then Class = '1:1 or 3:1 Segregation';
   End;
   *** Determine Origin of Marker based on Parental Data ***;
   If Test = 200 then Origin = 'Both Parents          ';
   If Test = 100 and AM = 100 then Origin = 'American Parent       ';
   If Test = 100 and F1x = 100 then Origin = 'F1 Parent';
   If Test =   0 then Origin = 'Unknown               ';
   If Test =   . then Origin = 'Unknown               ';

   *** Cross-check Classification and Origin ***;
   If Origin = 'Unknown               ' then Rescore = 'Yes';
   If Class = '3:1 Segregation       '
      and Origin NE 'Both Parents          ' then Rescore = 'Yes';
   If Class = '1:1 Segregation       '
      and Origin EQ 'Both Parents          ' then Rescore = 'Yes';
   If Class = 'Distorted 1:1         '
      and Origin EQ 'Both Parents          ' then Rescore = 'Yes';
run;


 *** 6. Tables for Segregation Patterns and Parentage at p=.05 ***;;


Data Mollie06; set Mollie05;
*** Print Out Summary Table ***;
Proc Tabulate Missing;
   Class Class Origin Rescore;
   Table (All Class),(All Origin),(All Rescore);
   Title4 'All of the Markers from Quantar';


*** Print Data by Segregation Classification ***;
Proc Sort; By Class Frequency;
Proc Print NoObs;By Class;
   PageBy Class;
   Var Primer MW Quality Frequency Origin Rescore F1x AM Sign11 Sign31;
```

```
   Title3 'Data Sorted by Classification';

*** Print Data by Frequency ***;
Proc Sort        Data = Mollie06; By Frequency;
Proc Print NoObs Data = Mollie06;
     Var Primer MW Quality Num Neg Plus Frequency Sign11 Sign31 ChiSq11
ChiSq31
           Prob11 Prob31;
       Format Frequency ChiSq11 ChiSq31 6.2 Prob11 Prob31 6.4;
     Title3 'Data Sorted by Marker Frequency';


*** Print Data by Primer for Rescoring ***;
Proc Sort Data=Mollie06; By Primer MW;
Proc Print Data=Mollie06 NoObs;By Primer;
   PageBy Primer;
   Var MW Rescore Quality Origin  Class GR F1x AM Sign11 Sign31
Frequency;
   Title3 'Data Sorted for Rescoring';
Run;


                        *** Output for mapmaker ***


      *** 7a. Suffix for Parental Origin for each marker ***;

Data Mollie07; Set Mollie06;
If Class01 Eq '1:1 or 3:1 Segregation' Then Suffix1 = 'U';
If Class Eq '1:1 Segregation' and Class01 = '1:1 Segregation' then
Suffix1 = 'o';
***In the above line 'o' is short for 'omit';
If Origin = 'Both Parents' then Suffix1 = 'B   ';
If Origin = 'F1 Parent' then Suffix1 = 'F  ';
If Origin = 'American Parent' and Class = '1:1 Segregation'
      then Suffix1 = 'omit';
If Origin = 'American Parent' and Class = 'Distorted 1:1'
      then Suffix1 = 'omit';
Else If Origin = 'American Parent' then Suffix1 = 'A';
*the above line is used as a check--any marker with a suffix = 'A' is
unwanted;
If Origin = 'Unknown' and AM = 100 and Class = '1:1 Segregation'
      then Suffix1 = 'omit';
If Origin = 'Unknown' and AM = 100 and Class = 'Distorted 1:1'
      then Suffix1 = 'omit';

If Origin = 'Unknown' and F1x = 100 and Class  = '1:1 Segregation'
      then Suffix1 = 'f';
If Origin = 'Unknown' and F1x = 100 and Class = 'Distorted 1:1'
      then Suffix1 = 'f';
Else IF Origin = 'Unknown' then Suffix1 = 'U   ';
run;


      *** 7b. Marker Suffixes for Seg. Patterns and Conf. Intervals***;
```

```
Data Mollie08; Length Suffix2 $ 18; set Mollie07;
If Class = 'Distorted 1:1' and Class01 = '1:1 Segregation' then Suffix2
= '1:1-01  ';
If Class = 'Distorted 1:1' and Class01 = 'Distorted 1:1'
      then Suffix2 = 'd1:1-01  ';

If Class = '1:1 Segregation' then Suffix2 = '1:1-05  ';
If Class NE '1:1 Segregation' and Class01 = '1:1 Segregation'
      then Suffix2 = '1:1-01  ';

If Class = '3:1 Segregation' then Suffix2 = '3:1-05  ';
If Class NE '3:1 Segregation' and Class01 = '3:1 Segregation'
      then Suffix2 = '3:1-01  ';

If Class = '1:1 or 3:1 Segregation' and Class01 = '1:1 Segregation'
      then Suffix2 = '1:1-05  ';
If Class = '1:1 or 3:1 Segregation' and Class01 = '3:1 Segregation'
      then Suffix2 = '3:1-01  ';

If Class = '1:1 or 3:1 Segregation' and Class01 = '1:1 or 3:1
Segregation'
      then Suffix2 = '3:1,1:1-05';


        *** 7c. Determining parentage of markers segregating
                        1:1 & d1:1 with one known parent ***;


If Suffix2 = '1:1-01  ' and F1x = 100 then Suffix1 = 'F';
If Suffix2 = '1:1-05  ' and F1x = 100 then Suffix1 = 'F';
If Suffix2 = 'd1:1-01  ' and F1x = 100 then Suffix1 = 'F';
If Suffix2 = 'd1:1-05  ' and F1x = 100 then Suffix1 = 'F';

If Suffix2 = '1:1-01  ' and Am = 100 then Suffix1 = 'omit';
If Suffix2 = '1:1-05  ' and Am = 100 then Suffix1 = 'omit';
If Suffix2 = 'd1:1-01  ' and Am = 100 then Suffix1 = 'omit';
If Suffix2 = 'd1:1-05  ' and Am = 100 then Suffix1 = 'omit';

*next series of commands are new;
If Suffix2 = '1:1-01  ' and Test = 0 then Suffix1 = 'U';
If Suffix2 = '1:1-05  ' and Test = 0 then Suffix1 = 'U';
If Suffix2 = 'd1:1-01  ' and Test = 0 then Suffix1 = 'U';
If Suffix2 = 'd1:1-05  ' and Test = 0 then Suffix1 = 'U';


        *** 7d. Discard markers originally segregating at 3:1
                        but now determined as 1:1 American ***;


If Suffix1 = 'A' and Suffix2 = '1:1-05' then Suffix1 = 'omit';
If Suffix1 = 'A' and Suffix2 = '1:1-01' then Suffix1 = 'omit';
run;
*next 2 data sets and commands help in calculating the number of
markers omitted;
Data Mollie08omitted; set Mollie08;
```

```
If suffix1 NE 'o' then delete;
drop g1-g100 check chisq11 prob11 chisq31 prob31 prob11
                  chisq1101 prob1101 chisq3101 prob3101
      primer quality mw plus neg i df frequency;
run;
proc sort; by class class01 rescore;run;


Data Mollie08chart; set Mollie08;
If suffix1 = 'o' then delete;
proc tabulate; class suffix1 suffix2; table (All suffix1),(All
suffix2); run;


                              *** 7d. Marker Sorting ***;


Data Mollie09; set Mollie08;
If Rescore = 'No' then Suffix3 = 'D';
      *D = Does not need rescoring;
If Rescore = 'Yes' then Suffix3 = 'R';
      *R = will need Rescoring;
If Suffix2 = '3:1-01  ' and Suffix1 = 'B' then Suffix3 = 'D';
If Suffix2 = '3:1-05  ' and Suffix1 = 'B' then Suffix3 = 'D';
run;
Proc Sort;
by Suffix3 Suffix2 Suffix1 primer MW;
run;


                        *** 8. Output for EXCEL/MAPMAKER ***;;


Data Mollie10; Length Marker $ 32; set Mollie09;
If Suffix1 = 'omit' then Delete;
Array G(88);
   Do I = 2 to 88;
      If G(I) = 0 then G(I) = 2; *marker absent;
      If G(I) = . then G(I) = 0; *       unknown;
   End;
      IF GR  = 100 then GR   = 1;
      IF GR  = 0 then GR  = 2;
      If GR  = . then GR  = 0;
      IF AM  = 100 then AM   = 1;
      IF AM  = 0 then AM  = 2;
      If AM  = . then AM  = 0;
      IF F1x = 100 then F1x  = 1;
      IF F1x = 0 then F1x = 2;
      If F1x = . then F1x = 0;
      marker = compress(primer||MW||quality);
      suffix = compress(Suffix1||Suffix2||Suffix3);
run;

Proc tabulate Missing;
   Class Class Origin Rescore;
   Table (All Class),(All Origin),(All Rescore);
```

```
   Title4 'Markers Left after SAS-Processing';
run;

Proc tabulate Missing;
      Class Suffix1 Suffix2 Suffix3;
      Table (All Suffix1),(All Suffix2),(All Suffix3);
      Title4 'Markers Left after SAS-Processing';
      Title6 'Suffix1 = Parents: A=American B=Both F=F1 U=Unknown';
      Title7 'Suffix2 = 1:1, etc= segregation pattern; 05 or
01=confidence level';
      Title8 'Suffix3 = rescore: D=don''t rescore R=rescore';
      Title9 ' ';
      Title10 ' ';
run;

*this exported file below can be used if joinmap will not to be used;
Data Mapmakerformat02; set Mollie10;
drop xmarker check primer Quality neg num I G89 G90 G91;
drop G1 df ChiSq11 Prob11 Sign11 ChiSq31 Prob31 Sign31;
drop G1 df ChiSq1101 Prob1101 Sign1101 ChiSq3101 Prob3101 Sign3101;
drop Frequency Test Rescore Class;
drop MW Origin Class01 Plus;
***The next line takes out the next steps in excel (easy to forget);
drop G2 G62 G68 GR F1x AM;
run;

Proc Export data=Mapmakerformat02
Outfile= 'C:\Thesis\ch42-less9s-SASoutput.xls' replace;
run; quit;


**********************************************************************
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**********************************************************************
**********************************************************************;


      *** 9.  Formatting the data for Joinmap according to
          tables 5, 6, & 7 on joinmap manual pp. 37 & 40 ***;


*deletes unneeded variables from SAS & leaves in some removed above for
mapmaker formatting;;
Data Joinmap01;
length marker $ 20 suffix1 $ 4 suffix2 $ 10 suffix3 $ 4 suffix$ 18;
set Mollie10;
xmarker = marker;
drop check primer Quality neg num I G89 G90 G91;
drop G1 df ChiSq11 Prob11 Sign11 ChiSq31 Prob31 Sign31;
drop G1 df ChiSq1101 Prob1101 Sign1101 ChiSq3101 Prob3101 Sign3101;
drop Frequency Test Class;
drop MW Origin Class01 Plus;
run;
**SEGb forces:
      a) 1:1 markers   - at 05 level      : as if   F1=100
      b) 1:1 markers   - at 01 level      : as if   F1=100
```

```
      c) distorted 1:1 - at 05 level      : as if   F1=100
      d) distorted 1:1 - at 01 level      : deleted (by prior coding)
      e) 3:1,1:1                          : as 3:1  (prior coding
                                          ensures this is appropriate)
      f) 3:1 regardless of parent scores  : as 3:1
**SEGc forces:
      a) 1:1 markers - at 05 level where F1 ne 100    : as if   AM=100
      b) 1:1 markers - at 01 level where F1 ne 100    : as if   AM=100
      c) distorted 1:1 - at 05 level where F1 ne 100: delete(redundant)
      d) distorted 1:1 - at 01 level                  : as if   AM=100
      e) 3:1,1:1                                   : delete  (redundant)
      f) 3:1 regardless of parent scores           : delete  (redundant)
**each data set will be appended to work.joinmapbcd to create a data
set containing all the desired iterations for each marker this
work.joinmapbcd will then be exported to excel;;

*bbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbbb;

Data Joinmap02; set Joinmap01; *order of following commands changed;
**********;
If Suffix2='1:1-05'  then SEGb = '<lmxll>';
If Suffix2='1:1-01'  then SEGb = '<lmxll>';
If Suffix2='d1:1-05' then SEGb = '<lmxll>';
If Suffix2='d1:1-01' then delete;
If Suffix2='3:1,1:1-05' then SEGb = '<hkxhk>';
If Suffix2='3:1-05' then SEGb = '<hkxhk>';
If Suffix2='3:1-01' then SEGb = '<hkxhk>';
Run;


*The follow code assumes all special SEGb assumptions were correct;;
**Also, ItSuffix is introduced to describe iteration types within the
marker name
  A blank ItSuffix means that the marker was 1:1 or 3:1 AND did not
need iteration recoding;;
Data SEGb; set Joinmap02;
If Suffix2 = '1:1-05' and F1x ne 1 then ItSuffix = '1:1-5R';
If Suffix2 = '1:1-01' and F1x = 1 then ItSuffix = '1:1-01';
If Suffix2 = '1:1-01' and F1x ne 1 then ItSuffix = '1:1-1R';
If Suffix2 = '3:1-05' and Rescore = 'No' then ItSuffix = '3:1-05';
If Suffix2 = '3:1-05' and Rescore = 'Yes' then ItSuffix = '3:1-5R';
If Suffix2 = '3:1-01' then ItSuffix = '3:1-01';
If Suffix2='3:1,1:1-05' then ItSuffix = '3:1-F';
*The only remaining cases are 1:1-05 markers
where F1x = 1, and for these, ItSuffix '_';
Array G(88) $;
Array H(88) $;
      If SEGb = '<lmxll>' then Do I = 2 to 88;
      If G(I) = 1 then H(I) = 'lm'; *marker present;
        If G(I) = 2 then H(I) = 'll'; *       absent;
      If G(I) = 0 then H(I) = '--'; *       unknown;
      End;
      IF SEGb = '<hkxhk>' then do I = 2 to 88;
      IF G(I) = 1 then H(I) = 'h-'; *marker present;
        If G(I) = 2 then H(I) = 'kk'; *       absent;
      If G(I) = 0 then H(I) = '--'; *       unknown;
```

```
    END;
rename SEGb = SEG;
marker = compress(xmarker||ItSuffix);
drop G1-G88 I;
run;
Proc tabulate;
      Class ItSuffix; Table (ItSuffix);
run;quit;

*ccccccccccccccccccccccccccccc;
a) 1:1 markers   - at 05 level where F1 ne 100        : as if   AM=100
b) 1:1 markers   - at 01 level where F1 ne 100        : as if   AM=100
c) distorted 1:1 - at 05 level where F1 ne 100        : as if   AM=100
d) distorted 1:1 - at 01 level                  : delete  (redundant)
e) 3:1,1:1                                      : delete  (redundant)
*The follow code assumes all special SEGc assumptions were correct;;
Data SEGc; set SEGb;
If Suffix2='1:1-01' and F1x ne 1 then SEGc = '<nnxnp>';
If Suffix2='1:1-05' and F1x ne 1 then SEGc = '<nnxnp>';
If Suffix2='d1:1-05' and F1x ne 1   then SEGc = '<nnxnp>';
IF SEGc = ' ' then delete;
ItSuffix = '1:1-A';
Array H(88) $;
      If SEGc = '<nnxnp>' then Do I = 2 to 88;
      If H(I) = 'lm' then H(I) = 'np'; *marker present;
        If H(I) = 'll' then H(I) = 'nn'; *       absent;
      If H(I) = '--' then H(I) = '--'; *       unknnown;
      If H(I) = 'h-' then H(I) = 'np'; *marker present;
        If H(I) = 'k-' then H(I) = 'nn'; *       absent;
      If H(I) = '--' then H(I) = '--'; *       unknown;
      End;
marker = compress(xmarker||ItSuffix);
drop I  SEG;
rename SEGc = SEG;
run;

*****************************************************************;
*****************************************************************;

*the next section goes off on a tangent: it tallies markers by their
likelihood of
       making it into a map of the F1 based on whether or not a 2nd or
3rd iteration
       of the segregation patterns/parent scores were created above;;

DATA joinmap03;  Length MarkerType $ 20; set SEGb;
If marker = xmarker and Rescore = 'No'  then MarkerType = '1:1 good,
p=.05';
      *A= Quite likely to work--1:1 with F1 origin at p = .05;
If ItSuffix = '1:1-01' then MarkerType = '1:1 good, p=.01';
    *B= Also quite likely to work--1:1 with F1 origin at p = .01;
If ItSuffix = '1:1-5R' then MarkerType = '1:1-05 rescore';
If ItSuffix = '1:1-1R' then MarkerType = '1:1-01 rescore';
      *C=these markers could have their parents rescored and maybe fit
into 1:1 F1;
If ItSuffix='3:1-05'  then MarkerType = '3:1, good p=.05';
```

```
If ItSuffix='3:1-01'  then MarkerType = '3:1, good p=.01';
      *C= might work--this marker is a 3:1 marker;
If ItSuffix='3:1-F' then MarkerType = '3:1 or 1:1';
If ItSuffix='3:1-5R'    then MakerType = '3:1, good p=.05';
      *D= only Slightly likely to work --this marker's origin/segr.
            pattern don't agree, but may work as an F1 origin;
Run;

Proc tabulate;
      Class MarkerType; Table (MarkerType);
run;quit;

*Appends data lines from SEGc and SEGd cases to SEGb into SEGbcd;;

Data Joinmapbcd; length marker $ 20; set SEGb;
Proc append base = joinmapbcd data = SEGc force;
*Proc append base = joinmapbcd data = SEGd force; run; quit;
Data joinmap04; set joinmapbcd;
drop H1 suffix1  suffix3 suffix;
drop /*GR F1x AM*/ Rescore xmarker; run;
Proc sort data=joinmap04; by SEG; run;
Proc Export data=joinmap04
Outfile= 'C:\Thesis\ch42-jmapformat.xls'
replace; run; quit;

****at this point the only markers without a suffix are
      1:1 markers that do not need to be rescored;

Data Translate01; set Joinmap04;
Array H(88) $;
If SEG = '<lmxll>' then Do I = 3 to 88;
      If H(I) = 'lm' then H(I) = '1'; *marker present;
      If H(I) = 'll' then H(I) = '2'; *       absent;
      If H(I) = '--' then H(I) = '0'; *       unknown;
      End;
IF SEG = '<hkxhk>' then do I = 2 to 88; *3:1 markers, special cases;
      IF H(I) = 'h-' then H(I) = '0'; *marker present--convert + to 0;
      If H(I) = 'kk' then H(I) = '2'; *       absent;
      If H(I) = '--' then H(I) = '0'; *       unknown;
    END;
IF SEG = '<npxnn>' then do I = 2 to 88; *3:1 markers, special cases;
      IF H(I) = 'np' then H(I) = '1'; *marker present--convert + to 0;
      If H(I) = 'nn' then H(I) = '2'; *       absent;
      If H(I) = '--' then H(I) = '0'; *       unknown;
    END;
drop SEG ItSuffix I H1;
run;
data translate02; set translate01;
*new; GR = H86; F1 = H87; AM = H88;
drop H86 H87 H88; run;
Proc Export data=Translate02
Outfile= 'C:\Thesis\ch42-jmaporder-mpmkrformat.xls'
replace; run; quit; ;


            ********** END OF PROGRAM; **********
```