7

Breeding American Chestnuts for Blight Resistance

Lisa M. Worthen, Keith E. Woeste, and Charles H. Michler
Hardwood Tree Improvement and Regeneration Center
U.S. Forest Service Northern Research Station
Department of Forestry and Natural Resources
Purdue University
715 West State Street
West Lafayette, IN 47907

Plant Breeding Reviews, *Volume 33* Edited by Jules Janick

I. INTRODUCTION

When Burnham, Rutter, and French (1986) published their proposal to use backcross breeding methods such as those used in annual crops to breed American chestnut trees that would be resistant to the chestnut blight disease, the prospect seemed the hopeful imaginings of dedicated few. In the 20 or so years since that publication, a nationwide effort involving thousands of volunteers, hundreds of backcross breeding experiments, many universities, and numerous government and private institutions has culminated in seed orchards that contain blight-resistant American chestnut hybrids. These hybrids will be the parents of seeds used for reforestation trials. While backcross breeding programs continue to incorporate new sources of resistance and new locally adapted American chestnut genotypes, chestnut breeders must prepare for the long-hoped-for return of American chestnuts to forests of the eastern United States and the appearance of American chestnuts in plantations.

Breeders face unusual challenges when seeking to reintroduce populations to their former habitats or to supplement wild populations that may no longer be viable. Conservation breeders may use traditional breeding methods, but unlike crop breeders, they must release the products of their program into environments over which landowners typically will exercise little control. Indeed, the goal is to return healthy individuals back into the wild—into environments marked by dynamic change and nonuniformity. In effect, conservation breeders are seeking to reverse the typical work flow of breeding. Crop breeders typically incorporate wild germplasm into cultivated or domesticated lines and seek to improve the predictability of yield by manipulating the genetics and culture of the crop. American chestnut breeders introgress genes from a cultivated species into a wild relative with the hope that their selections will thrive in unmanaged or minimally managed stands. Conservation breeding of forest trees also differs from traditional forest tree breeding in that forest geneticists select their releases for optimal performance in even-aged plantations established within discrete zones of adaptation, a relatively circumscribed environment compared to the goal of restoring a species to its entire former range.

This chapter examines how outcomes of historical and current American chestnut breeding may affect reintroduced chestnut populations. What levels of genetic diversity and family structure will characterize reintroduced populations? How will reintroduced populations survive, mate, and spread? What will be the fate of native American chestnut genes? Twenty years after Burnham, Rutter, and French's

seminal publication in this same journal, an assessment of past breeding efforts and an examination of the genetic issues affecting future reintroductions is warranted.

II. CHESTNUT GENETICS

The genus *Castanea* is comprised of three sections and seven species (Johnson 1988). Four species—*Castanea mollissima* Blume (Chinese chestnut), *C. seguinii* Dode. (Seguin chestnut), *C. crenata* Sieb. and Zucc. (Japanese chestnut), and *C. henryi* Skan (Chinese chinquapin)—in eastern Asia. The European chestnut, *C. sativa* Mill., is native to western Asia and southern Europe, and *C. dentata* (Marsh.) Borkh. (American chestnut) and *C. pumila* Mill. (American chinquapin, often divided into subspecies Ozark and Alleghany chinquapin) occur in eastern North America.

Genetic variation, population structure, and phylogenetic relationships within and among *Castanea* species have been analyzed using a variety of marker systems (Villani et al, 1991; Huang et al. 1994, 1998; Lang and Huang 1999; Dane et al. 1999, 2003; Kubisiak and Roberds 2003; Tanaka et al. 2005; Lang et al. 2006, 2007; Wang et al. 2006; Han et al. 2007). Sequence-based phylogenetic analysis of the variable *trn*T-L-F region of *Castanea* cpDNA showed *C. crenata* the most basal species and placed the Chinese species in a monophyletic clade with the North American and European species as a sister group (Lang et al. 2006). In general, *Castanea* species show high levels of genetic diversity at neutral loci, little population structure, and partition most genetic diversity within populations—population genetic traits shared by many other long-lived, outcrossing forest trees (Petit and Hampe 2006). In North America, *Castanea* species were driven southward to one or two southern glacial refugia during the Wisconsin glacial maximum 18,000 to 20,000 years ago. *C. dentata* greatly expanded its range after the glacial retreat about 10,000 years ago, spreading northward along the Appalachian ridge (Delcourt and Delcourt 1984; Huang et al. 1998; Delcourt 2002) and establishing itself as a dominant species in 800,000 km^2 of eastern forest (Braun 1950).

C. dentata, like all *Castanea* species, is diploid ($2n = 2x = 24$) and will easily hybridize with its congeners, although offspring from some combinations suffer from low vigor or male sterility (Jaynes 1975). It is monoecious, primarily wind pollinated, and generally bears three heavy seeds per cupule. The American chestnut is an obligate outcrosser with a genetic self-incompatibility system that prevents self-fertilization

(McKay 1942). The self-incompatibility system appears to be gametophytic because chestnut has binucleate pollen grains, and multiple pollen tubes penetrate to the base of the style prior to fertilization (McKay 1942; Brewbaker 1957; Burnham et al. 1986). Chestnut flowers become receptive in a pattern described by Stout (1928) as duodichogamy: Staminate flowers borne on long, slender catkins mature first, followed by pistillate flowers, and, last, staminate flowers on bisexual catkins. Under favorable conditions, American chestnut trees reach reproductive maturity between 12 and 18 years of age and mast abundantly every year (Buttrick 1925; Weeks et al. 2006). American chestnut seedlings have been shown to outgrow co-occurring hardwoods under high light conditions, but they can also survive for prolonged periods in shaded understories (Paillet and Rutter 1989; McCament and McCarthy 2005). Records indicate that in some locations, chestnut was a medium-size tree, but on favorable sites, American chestnut trees grew to 37 m tall and 1.5 m in diameter (Buttrick 1925; Illick 1928; Braun 1950). The largest recorded specimen was about 5.2 m in diameter (Detwiler 1915).

The preblight population genetics of the American chestnut must be inferred from knowledge of postblight American chestnut populations and other forest trees with similar life history characteristics. Analyses using allozymes have shown that *C. mollissima* has the highest population mean genetic variability of all the members of the genus [total genetic variability (H_T) = 0.321, H_{exp} = 0.311; Lang and Huang 1999] and *C. dentata* has the lowest mean variability at isozyme loci (H_T = 0.214, H_{exp} = 0.167; Huang et al. 1998). Pigliucci et al. (1991) reported an intermediate mean expected heterozygosity for *C. sativa* (H_{exp} = 0.24 − 0.27). Kubisiak and Roberds (2003) employed 6 microsatellite and 19 random amplified polymorphic DNA (RAPD) loci to examine 17 populations of root crown collar sprouts of *C. dentata*. They used Nei's (1978) gene diversity (h) and calculated a mean genetic diversity across RAPD loci (h = 0.226) that, barring differences in marker systems, is similar to the diversity for American chestnut reported by Huang et al. (1998). Both reports are well within genetic diversity limits of similar forest tree species (Hamrick, Godt, and Sherman-Broyles 1992). Higher-than-average heterozygosities have been found in postblight American chestnut populations, leading some to speculate on the role of heterozygote advantage in the species' remarkable growth (Stilwell et al. 2003).

The spatial genetic structure characteristic of wind-pollinated, self-incompatible forest tree species is most often negligible or weakly present at only very close distances (Berg and Hamrick 1995; Streiff et al. 1998). Pierson et al. (2007) used minisatellite band sharing to calculate the differentiation between sampling plots in a large woodland

of naturalized American chestnut 600 km west of the species' native range. They determined that no differentiation among sampling plots had occurred ($F_{ST} < 0.031$) and that gene flow was not restricted within the woodland. Kubisiak and Roberds (2003) reported a similar differentiation value ($G_{ST} = 0.036$) and concluded that preblight populations of *C. dentata* followed a pattern consistent with the hypothesis of a single metapopulation where genetic drift was a dominant force in structuring populations. Conversely, Huang et al. (1998) reported a threefold higher measure of genetic differentiation among American chestnut populations ($G_{ST} = 0.110$) and determined that four large metapopulations existed within the range of American chestnut. Both the study of Huang et al. (1998) and Kubisiak and Roberds (2003) reported a cline in allele frequencies along an axis from the southwestern to the northeastern limits of the species' natural range. Differences in their findings may have been the result of differences in sampling strategies. Kubisiak and Roberds (2003) excluded individuals with a *C. pumila* chloroplast type, whereas the study of Huang et al. (1998) did not attempt to exclude cryptic hybridization and thus may have inflated between-population differences. Also, Kubisiak and Roberds (2003) sampled continuously down the range of *C. dentata*, whereas Huang and colleagues sampled fewer and more outlying populations. In *C. sativa* and *C. dentata*, allele frequencies showed differentiation over altitudinal and edaphic clines, suggesting the possibility of local adaptation across the wide range of the two species (Villani et al. 1991; Kubisiak and Roberds 2003).

In summary, American chestnut was once a dominant canopy tree over a wide range of edaphic and moisture conditions (Russell 1987). It was a good competitor in disturbed areas with high light levels but also could persist for many years in the forest understory, leading some to hypothesize that it is adapted to low-light conditions (McCament and McCarthy 2005). Like many wind-pollinated, heavy-seeded forest trees, it probably combined the apparently paradoxical traits of high pollen gene flow and local adaptation (Petit and Hampe 2006) resulting in little genetic differentiation between locally adapted populations. The species now exists in the understory of forests across its historical natural range as root collar sprouts that often do not reach reproductive maturity before succumbing to chestnut blight disease.

III. CHESTNUT BLIGHT

Chestnut blight disease is incited by the fungus *Cryphonectria parasitica* (Murr.) Barr. The fungus causes a girdling canker on stems and branches

of American chestnut that are lethal to the trees (Roane et al. 1986). The disease was introduced to North America on Japanese chestnut trees (Cunningham 1984; Anagnostakis 1992) and was discovered at the New York Zoological Gardens in 1904 (Merkel 1906). By 1950, the disease had spread across the entire range of the American chestnut, eliminating it as an overstory tree in eastern forests (Newhouse 1990). An estimated 4 billion trees were killed by chestnut blight disease, resulting in significant changes in forest communities and communities of people that depended on the tree for food, timber, and livestock feed (Russell 1987). Blight-killed trees often sprout from the root crown collar. It has been shown that root systems are weakened by the disease and produce fewer sprouts with each cycle of resprouting and dying (Griffin 2000). The prolific root crown collar sprouting of American chestnut provides a continuous supply of susceptible host tissue, a continuing reservoir of fungal inoculum in the forest, and a source of genetic diversity for chestnut conservation.

Chestnut blight is generally not lethal to the Asiatic species of chestnut, although a range of resistance among *C. mollissima* cultivars has been reported (Huang et al. 1996), and *Castanea sativa* and *C. pumila* will both contract and succumb to chestnut blight infection (Jaynes 1975). American chestnuts are generally accepted to be fully susceptible to chestnut blight fungus, although the presence of trees within the native range that have survived blight, and evidence that grafts from blight survivors show relatively high levels of resistance when inoculated with *Cryphonectria parasitica*, have led some to hypothesize some level of blight resistance in American chestnuts (Jaynes 1975; Griffin et al. 1983). Hybrids between susceptible American chestnuts and resistant chestnut species have intermediate levels of resistance (Clapper 1952).

In Europe, *Castanea sativa* suffered severe damage in the 1930s as the result of infection by the chestnut blight fungus, but populations have regenerated in recent decades. This recovery was due to the emergence and spread of naturally occurring hypovirulent strains of *Cryphonectria parasitica*, along with the deployment to chestnut growers of hypovirulent strains propagated in vitro (Grente and Sauret 1969; Grente and Berthelay-Sauret 1978). Hypovirulence is widely used in Europe to reduce mortality of *C. sativa*; however, hypovirulent strains of *C. parasitica* have not shown any degree of success at the population level in the recovery of American chestnut (Milgroom and Cortesi 2004), probably because there are more mycelial compatibility groups in North America than in Europe (Anagnostakis et al. 1986; Double and Macdonald 2002).

Early breeding results demonstrated that F_1 American $\times$ Chinese hybrids of above-average resistance backcrossed to Chinese chestnut

produced a high (>75%) percentage of resistant progeny, leading to the conclusion that inheritance of blight resistance may be under oligogenic control, perhaps conditioned by as few as two partially dominant genes (Clapper 1952). Standard deviations of canker size in F_2 populations were compatible with models for one or two incompletely dominant genes controlling blight resistance, based on Wright's method for estimating the number of factors controlling a segregating trait (Falconer 1960; Hebard 2006). A genetic map of American chestnut based on RAPD and restriction fragment length polymorphism (RFLP) markers was published in 1997 (Kubisiak et al. 1997), and the genetic map of *C. sativa* was published in 2001 (Casasoli et al. 2001). Both maps described 12 linkage groups presumably corresponding to the 12 chromosome pairs of *Castanea*. Initially, seven genomic regions (QTLs) were found to affect blight resistance in an F_2 population derived from a singe resistance source; three of the QTL held up after further investigation (Kubisiak et al. 1997). Reanalysis of the original F_2 mapping population (after the addition of hundreds of markers and the removal of simple sequence repeat [SSR] contaminants) and analysis of a BC_1 population derived from a separate resistance source confirmed the presence of three loci (*Cbr1*, *2*, and *3*) conditioning resistance to chestnut blight. It is unknown whether the genes are associated with two or three linkage groups (Kubisiak et al. 1997; Sisco et al. 2005).

IV. BLIGHT-RESISTANCE BREEDING

A. Historical

Efforts to breed blight resistance into American chestnuts began in the late 1920s, when extirpation of the species was believed imminent. Scientists with the United States Department of Agriculture (USDA) began crossing American chestnuts to resistant species to make F_1 hybrids, but the program was abandoned when blight resistance in the F_1 hybrids proved insufficient (Clapper 1952; Jaynes 1974). During the 1930s, A. H. Graves began an American chestnut breeding program utilizing various crosses of American, Chinese, and Japanese chestnuts at the Connecticut Agricultural Experiment Station; the program was continued into the 1980s by R. A. Jaynes (1978). Burnham (1982) suggested that if blight resistance was under oligogenic control as suggested by Clapper (1952), introgressing genes from resistant Asiatic species should be possible by making hybrids and backcrossing to the recurrent American parent only the most resistant individuals as determined by a

disease challenge. The American Chestnut Foundation (TACF), a nonprofit organization, took up this task. A BC_1 [(Chinese × American) American] chestnut tree from the early USDA breeding program showed unusually good form and blight resistance (Diller and Clapper 1969). This individual, eventually called the 'Clapper' hybrid, was used to begin the American Chestnut Foundation breeding program; in fact, the most advanced backcross hybrids in the breeding program today are descendants of the original USDA 'Clapper' hybrid (Clapper 1963; Hebard 2004). A second unrelated BC_1, called the 'Graves' hybrid, entered the breeding program soon after, and a third *C. mollissima*, 'Nanking', was used as the parent of 20 recurrent F_1 lines in 1989. As of 2004, TACF's Meadowview Research Farms had over 17,000 trees at various stages of breeding planted on more than 52 ha of land (Sisco 2004). Most of these trees are descendants of the 'Graves' and 'Clapper' hybrids, such that two sources of resistance (from the 'Graves' Chinese grandparent 'Mahogany' and the 'Clapper' Chinese grandparent PI 34517) provide the foundation for most of the blight resistance in the breeding program. In addition, 34 Chinese or Japanese chestnut trees have been used in smaller numbers as sources of resistance, with some sources as far advanced as the BC_3 generation (Hebard 2006).

Soon after its inception, the TACF organized into state chapters that sought out living American chestnut trees with the goal of introducing locally adapted genotypes into each state's breeding population (Burnham et al. 1986). State chapters pollinated these "mother" trees with advanced backcrosses of either 'Clapper' or 'Graves' sources of resistance; alternatively, chapters were encouraged to find unique sources of resistance and make F_1 crosses. As of 2003, there were about 375 maternal lines in the TACF breeding program; the number is expected to approach 500 across all state chapters as programs mature (Hebard 2002).

Concerns about the fate of artificially regenerated populations of blight-resistant American chestnut center around seedling survival and the potential breakdown of blight resistance. The silviculture of American chestnut has an essential role in reintroduction because it informs where the products of the breeding program can survive. Research is under way to determine seedling requirements for light and nutrients, site conditions that favor chestnut growth, and the efficacy of bare-root and direct-seeding planting methods (e.g., Jacobs and Severeid 2004; Jacobs et al. 2004; Selig et al. 2005). Even in the most favorable growing conditions, however, the survival of reintroduced trees will remain the outcome of complex interactions between a pathogen and a host with a hybrid genome. It has been suggested that resistance itself needs to be variable, a goal that could be achieved by including more sources of

resistance into breeding populations (Ellingboe 2001 as cited by Irwin 2003).

Management of and selection in the *C. dentata* hybrid breeding program is primarily targeted toward the recovery of American chestnut form and ecological behavior following the initial hybridization to Chinese chestnut. On average, 94% of the genome of all third-backcross hybrids and their descendants comes from American chestnut; 6% is inherited from the Chinese chestnut ancestor. The *C. dentata* genomic complement in the BC_3 generation could be smaller, however, if resistance genes are inherited in large blocks, or larger if selection is practiced against those *C. mollissima* alleles that are not responsible for conferring resistance to chestnut blight. Diskin and others (2006), using an index compiled from 24 leaf, stipule, twig, and bud morphological traits, determined that the complement of American genome in the BC_3 generation was not significantly different from expected under additive gene action. They further showed that species assignment based on measurements of diagnostic morphological traits would classify BC_3 hybrids as American chestnuts. In other words, most BC_3s in their study were morphologically indistinguishable from *C. dentata*. Although these results are promising, many traits with ecological and economic significance, such as growth rate and form, were not investigated, and bear heavily on the success of reintroduced American chestnut hybrids.

B. Seed Orchard Design and Forest Trials

A major goal of the TACF is to propagate American chestnut seed orchards that conserve as much genetic diversity as possible (Burnham 1986; Hebard 1994). It has been suggested that in order to retain 95% chance of capturing most alleles in a maternal BC_3 parent, at least 9 highly resistant BC_3F_2 offspring are needed (Hebard 2002). Using the assumptions of random mating and three-gene control of blight resistance, 1,080 BC_3F_2 trees per BC_3 line need to be grown to ensure with 99% confidence that at least 9trees will be fully resistant (i.e., homozygous for three genes, Hebard 1994). If 8 trees grow well for every 10 seeds planted, then about 1,350 nuts need to be planted from each BC_3 parent to obtain 8e highly resistant BC_3F_2 progeny, or 150 nuts for each desired BC_3F_2 (Hebard 2002).

Seed orchards containing between 20 and 30 BC_3 lines have been planted in Virginia and Pennsylvania, and they are expected to begin producing large numbers of open-pollinated BC_3F_2 offspring by 2010. Guidelines for implementing replicated forest trials of BC_3F_2 families

were established by TACF in 2004. The three main objectives of forest trials are to determine: (1) to what degree BC_3F_2 trees resemble American chestnut in a forest setting and to what degree *C. mollissima* characters (other than blight resistance) may remain; (2) to what degree BC_3F_2 trees are resistant to blight; and (3) how long resistance persists in BC_3F_2 plantations (Steiner et al. 2004).

Test plantations will be on naturally forested sites temporarily devoid of trees (such as after a recent harvest), on soils considered suitable for American chestnut, and in close proximity to existing sprouts of American chestnut. Tests will contain American chestnuts as controls, Chinese chestnuts as controls, a core set of at least five BC_3F_2 families common to all test plantations, and additional BC_3F_2 families from regional breeding programs, as space allows. Planting will be in a completely randomized design (Steiner et al. 2004). When firm conclusions are possible regarding the relative performance of BC_3F_2 hybrids and Asian chestnuts, the Asian chestnuts may be removed from the plantations to eliminate their genes from the pollen pool.

V. POPULATION GENETICS OF HYBRID CHESTNUT REINTRODUCTION

The goal of the American Chestnut Foundation is not only to produce a blight-resistant American chestnut hybrid but "to bring blight resistance into wild, naturally regenerating populations of *Castanea dentata* in Appalachian forests and, by doing so, restore the species to its former role" (Steiner et al. 2004). Chestnut's "former role" includes its important economic uses, which should be complementary to its ecological and conservation values (Rajora and Mosseler 2001). Natural regeneration from planted trees, with accompanying natural selection and the potential for hybridization with native chestnut, is necessary for achieving this goal (Irwin 2003).

A. Small Population Size

The first obstacle to overcome in artificial regeneration is the establishment of populations of sufficient size to allow natural selection to effect evolutionary adaptation in the species (Irwin 2003). In general, a large number of founders are needed to avoid genetic sampling (founder) effects that cause introduced populations to have fewer alleles per locus relative to native or reference populations (Wright 1931; Ellstrand and Elam 1993). Genetic diversity in postfounder populations is often reduced further by

random losses of alleles through genetic drift and a genome-wide increase in homozygozity due to inbreeding, the result of repeated generations of mating in a finite population. Genetic drift and inbreeding reduce diversity more severely in small populations than in large ones because the magnitude of genetic drift is inversely proportional to the effective population size, N_e (Wright 1931; Kimura 1955). Inbreeding can also unmask and increase the frequency of deleterious rare alleles, leading to a further reduction in genetic diversity and population fitness. Falconer and Mackay (1996) presented equations that indicated populations with few alleles per locus and high homozygosity will have lowered additive genetic variance (although Ellstrand and Elam (1993) review cases where small populations may have larger additive genetic variation than large ones). As a consequence, small populations are expected to show reduced ability to adapt to adverse environmental pressures, and they are more likely to decline toward extinction (Nei et al. 1975; Ellstrand and Elam 1993).

The size a population must be in order to overcome the diversity-reducing effects of drift and inbreeding depends heavily on the environment and on the organism and its genome, which, taken together, can be viewed as "an integrated ecological strategy involving many trade-offs" (Petit and Hampe 2006). Attributes of the ecological strategy include life history and mating system, allelic diversity, DNA mutation rates, and genetic load. *Castanea* species, like many forest trees, are obligate outcrossers that employ a genetic incompatibility system that inhibits mating between closely related individuals (McKay 1942; Brewbaker 1957). A small population of chestnut trees consisting of only closely related individuals will produce few or no seeds, seeds may have low germination rates, and seedlings will generally lack vigor (McKay and Crane 1939; McKay 1942; Jaynes 1974). Byers and Meagher (1992) showed that small populations of species with self-incompatibility had higher variance for number of mates and fewer available mates, lower seed set, and increased variation in seed set relative to self-compatible populations. Large variance in reproductive success can lead to much lower N_e than would be predicted from census size alone (Falconer and Mackay 1996). Lifelong accumulation of somatic mutations results in a high genetic load, increasing the susceptibility of long-lived species to inbreeding depression. While mutation rates have not been directly studied in *Castanea*, most forest trees have relatively low mutation rates that compound the effects of inbreeding and drift (Petit et al. 2006).

Conversely, several attributes of chestnut's ecological strategy tend to reduce the dire genetic consequences associated with small populations, including the species' long life and outcrossing mating system.

Chestnuts also tend to have high allelic diversity at neutral loci (Kubisiak and Roberds 2003; Pierson et al. 2007). Stilwell et al. (2003) used genotypes at seven polymorphic allozyme loci to correlate heterozygosity with growth rate in stump sprout populations of *C. dentata*. They found a significant excess of heterozygotes (heterozygous at one or more loci) in the fast-growth category and concluded that a slight growth advantage for heterozygous genotypes may further maintain high levels of diversity in chestnut populations. High rates of pollen flow among populations reduce the risk of inbreeding. Rates of pollen flow and pollen dispersal data have not been published for American chestnut, so predictions must be based on indirect evidence. Chestnut trees are primarily wind pollinated, with small pollen grains (14–18 microns in *C. sativa*) that can travel long distances in the air (Fernandez-Lopez and Alia 2000). Pollen from wind-pollinated forest tree species has been shown to travel up to 100 km (Dow and Ashley 1998; Buiteveld et al. 2001; Petit and Hampe 2006). Erikkson et al. (2005) reported that pollinations of *Castanea sativa* dropped below 1% at distances of 2 km or more, and Cook and Forest (1979) observed that *C. dentata* usually required another tree within 100 m to set seed. They hypothesized that this relatively short distance was due in part to the presence of leaves in the forest and on the chestnut trees themselves when pollen is released in June. Thus, separated trees or populations can intermate provided they are close enough and share similar phenology. In addition to pollen movement, chestnut seeds are often dispersed short distances by small mammals, which tend to consume chestnuts or scatterhorde them close to the maternal tree (Steele et al. 2005). Blue jays (*Cyanocitta cristata*) have been shown to transport acorns 1 km or more in their specialized esophagus and have been credited with the rapid northward expansion of fagaceous trees in North America at the end of the last glacial maximum (Johnson and Webb 1989). Russell (1987) also reported an observation of blue jays dispersing American chestnut seeds; it is reasonable to assume they would again fill such a role in reintroduced populations. Long-distance dispersal by jays and other animals provides a mechanism whereby new genotypes could appear within pollination range of formerly isolated populations. While relatively few chestnut seeds will be dispersed long distances, mature chestnut trees have high fecundity, bear seeds in most years, and have multiseeded fruits, all of which increase the probability of long distance dispersal. Finally, to avoid potential problems associated with small and isolated populations, the TACF is planning many independent introductions of blight-resistant chestnut trees and seeds, a practice that has been shown to facilitate dispersal and population connection (Petit et al. 2004).

It is difficult to predict the factors that will most influence the genetic diversity and heterozygosity of regenerated American chestnut populations over many generations, in part because the species' rapid decline and mortality in response to chestnut blight disease precludes a full understanding of chestnut's preblight population genetics within its natural range. It is unknown if surviving American chestnuts represent a nonrandom sample of preblight populations or instead reflect a selective sweep at certain genomic regions. In an effort to determine how the population genetics of *C. dentata* has been affected by the abrupt change in life history caused by the chestnut blight disease, Stilwell et al. (2003) measured four populations of *C. dentata* for several growth-related traits and genotyped them at seven polymorphic allozyme loci. All loci showed more heterozygotes than expected, and three loci were significantly more heterozygous than expected. The observed excess of heterozygotes was also manifested in a significantly negative inbreeding coefficient ($F_{IS} = -0.2216$). The authors concluded that the slight growth advantage observed in heterozygotes caused an enrichment in heterozygosity over time and that chestnut blight disease significantly influenced the population genetics of American chestnut by halting sexual reproduction and recruitment of new seedlings, processes that would bring the population back toward Hardy-Weinberg equilibrium.

Examples of chestnut introductions may provide insight to the genetics of the reintroduced hybrid American chestnut. The largest and best-studied example is a population located in west-central Wisconsin, 600 km west of American chestnut's natural range. Ten American chestnut trees were planted there in the 1880s, and the stand now consists of about 5000 individuals on 20 ha of woodland (Paillet and Rutter 1989). Chestnut blight was first reported in the stand in 1987, and the population is currently in decline. Pierson and others (2007) used 84 minisatellite markers present in the founder trees to investigate diversity in the postfounder population. They found that no rare alleles had been lost and that heterozygosity in the postfounder population was slightly higher than in the founder population, results the authors attributed to chestnut's mating system, which favors unrelated matings, and to rapid expansion in the postfounder population. There was little differentiation among distant plots within the stand, suggesting high levels of gene flow. Additionally, they found seven alleles in the offspring that were not present in the founders, indicating a minisatellite mutation rate between 10^{-2} and 10^{-3} (Pierson et al. 2007).

Results from the Wisconsin stand concur with several studies showing that decreases in diversity during forest tree population expansion may be less drastic than formerly assumed. Daubree and Kremer (1993) found

identical levels of overall diversity in the introduced range (France and Germany) and the native range (eastern North America) of *Quercus rubra*. In European beech, an increase in heterozygosity occurred concomitantly with a major invasion during the Holocene (Petit et al. 2001). Still, informed precautions are warranted in chestnut reintroductions to ensure that the maximum reasonable amount of genetic diversity is captured. In an investigation of northern red oak, a species with similar life history characteristics as chestnut, Steiner (2006) suggested that selective forces acting on growth rate operated on a local "microsite" scale instead of a predictable geographic pattern and recommended that blight-resistant trees used to restore wild populations should be derived from local American chestnuts. Additionally, Kubisiak and Roberds (2003) showed that, although 95% of chestnut's neutral genetic diversity can be captured by sampling within any one population, populations have significantly different allele frequencies. In fact, the highest levels of neutral gene diversity and the greatest number of rare alleles were found in the southwestern portion of the American chestnut's natural range. Efforts to capture genes from this area (hypothesized to be a glacial refugium for American chestnut) and incorporate them into the American chestnut breeding program have resulted in several grafted mother-tree orchards with many accessions from the southwestern portion of the range (Alexander 2005).

Several important knowledge gaps reduce our ability to predict the success of reintroduction efforts. Genetic diversity at neutral loci may not adequately reflect diversity for adaptive and quantitative traits; thus, American chestnut heterozygosity may be overestimated. Indeed, the results of Stilwell et al. (2003) indicate that levels of heterozygosity seen in postblight American chestnut populations may be inflated beyond levels of heterozygosity that would occur in natural populations unaffected by chestnut blight. The effect of strong selection pressure on the regions of the genome that influence resistance to *Cryphonectria* is not known. The Wisconsin stand has only recently come under pressure from chestnut blight disease, whereas populations introduced within the native range will be planted into areas with existing populations of blight fungus. Although reintroduced trees are expected to be resistant to chestnut blight, variability in resistance among genotypes and variability in different fungal strains may influence the genetics of reintroduced populations. Because reintroduced chestnut populations may require an adaptive response at rather few loci (two or three loci are hypothesized to control blight resistance in chestnut), the population size needed to maintain genetic variability might be extremely large (Lexer et al. 2005; Willi et al. 2006). Willi and

others (2006) suggested that effective population sizes in the low thousands may be sufficient to maintain adequate adaptive potential in most cases.

B. Establishment, Colonization, and Expansion of Forest Tree Populations

Once planted, reintroduced populations must colonize new areas to expand and establish in the landscape and to move their genes out of the population while receiving new ones. In forest trees, dispersal events and the flow of genes generally occur at two distinct phases of the life cycle: pollination and seed dispersal (Petit 2004). The relevant difference between the two mechanisms of dispersal is that a pollen grain can transmit its genes only if it lands on a stigma, implying that there must be at least one adult plant already present. Thus, pollen can link existing populations or individuals through only immigration. Seeds, however, can colonize new areas where the species is not already represented. The result of colonization by seed is that the range of a species moves in one or more directions away from the introduced population, creating a leading edge and trailing edge (Petit 2004).

Three successive stages can be seen for any introduced population: arrival, establishment of a self-sustaining population, and integration, where ecological links are forged with existing or other introduced populations (Vermeij 1996). In the case of American chestnut reintroduction, arrival typically will occur when a number of trees are planted in multiple cleared areas of forest or onto old fields. It has been noted in other woody species that a lag time occurs between arrival of an introduced population and an increase in numbers that indicates a self-sustaining population; the mean lag time for introduced trees in Germany was 170 years (Sax and Brown 2000). Lag times are expected because of the long juvenile period of trees and due to early-stage exponential growth but can be exacerbated by competition with other woody species, unfavorable environmental conditions, or the need for adaptation to occur before further spread (Richardson 1998). As mentioned, the Wisconsin stand of naturalized American chestnut was started from 10 individuals in the 1880s; 127 years later, the stand included more than 5000 chestnuts. This must be considered an example of a successful expansion after the introduction of a tiny founder population. An introduction outside of a species' native range, however, may not recapitulate important ecological processes within the native range; the Wisconsin stand presented an assemblage of co-occurring trees, herbivores, dispersers, and pests (*C. parasitica* was not present

when the stand was established) different from that of the eastern forests where American chestnut hybrids will be reintroduced.

Considerations for the establishment phase are centered on seedling survival to reproductive maturity and production of viable seeds by adults. Silviculture experiments will guide planters as to site preparation, spacing, and care of seedlings (Jacobs 2007 and references therein). The fecundity of forest trees allows for intense selection to occur on germinated seedlings; few seedlings will survive to adulthood. Suitable sites for reintroduction traditionally have been identified based on the preferred location of adults, but adult trees have a much broader niche than seedlings (Poorter 2007). It will be important to determine the biotic and abiotic site characteristics that favor seedling survival and establishment —chestnut's "regeneration niche" (Grubb 1977)—and consider locating introduction points near such sites. The immediate regeneration niche for most hybrid chestnuts will be afforested sites (Jacobs 2007). Chestnuts likely will be planted into prepared and managed areas (e.g., mine reclamation sites; Jacobs et al. 2006) both within and outside the species' native range. The identification of factors that contribute to the natural regeneration and establishment of seedlings in association with, but outside of, these plantations will be an important area of research. Chestnut trees that survive to reproductive maturity and flower must be pollinated by unrelated individuals to avoid Allee effects (Allee 1938). Often discussed in animal ecology, but just as applicable to forest trees, Allee effects are the cyclical negative effects on population fitness and growth caused by too few individuals in a population of a species that benefits from the presence of conspecifics. Allee effects are manifested most severely in obligate outcrossing species, such as American chestnut, where a minimum population size is required for successful reproduction; if unrelated individuals are too rare, population growth may become negative. Fewer individuals mean fewer successful reproductions, individuals become even rarer, and population growth decreases further, in a cycle that can repeat until a local extinction occurs (Stephens and Sutherland 1999). Populations of American chestnuts must be reintroduced in a way that favors flower production and pollen dispersal to unrelated individuals to avoid a scenario where pollen limitation restricts seed production and hence the growth of a newly established population (Koenig and Ashley 2003). In the case of American chestnut, unrelated individuals or families will be planted together to minimize this pollen limitation, although differential survival and reproduction could result in reintroduced populations being more related than is desired.

Colonization of new territories occurs when seeds from the founder population are dispersed over a long distance in one or more directions. Paleoecological evidence suggests that even large barriers (such as lakes and mountain ranges) have posed few obstacles to colonization by many forest tree species (Clark et al. 1998). In addition, newly occupied habitats seem to favor seed dispersal relative to mature forests. The main disperser of European oaks, the European jay, is known to disperse more acorns, and more distantly, in open areas than in dense woodlands (Bossema 1979). Oaks in open areas have been shown to reach reproductive maturity in a fraction of the time of the same species in dense woodlands (20 years versus 30 to 45 years; Jensen and Nielsen 1986). It stands to reason that chestnuts, a prized food source of birds and small mammals, will be moved out of reintroduced plantings. What typically develops is called a seed shadow, where few seeds are dispersed long distances and increasingly more seeds are dispersed closer to the source population (Petit et al. 2004; Cousens et al. 2008). As newly dispersed seedlings grow and reproduce, family structure appears around the maternal tree that founded the new colony. Simulations have shown that these founder events eventually form a mosaic of patches over the landscape, with each patch having reduced diversity but all patches maintaining collectively a large share of diversity (Petit et al. 2001). Heterozygosity may be higher in these new populations relative to older populations, possibly leading to increased individual fitness and establishment success. For example, if a tree that was established by long-distance dispersal is completely self-incompatible and long-distance pollen flow is rare, the tree will produce few seeds (due to pollen limitation), but the seeds that are produced would all be sired by distant and distinct fathers. Assuming that pollen immigration is independent of target (sink) size, the rate of external gene flow would indeed be highest in the first generations (Petit et al. 2004). Founder effects are expected to be further attenuated by the long juvenile period of chestnut trees; the long period before maturity permits more seeds to arrive in a newly colonized patch (Austerlitz et al. 2000), assuming the regeneration niche lasts as long as the juvenile period of the chestnuts.

C. Monitoring Introduced Populations

Reintroduced populations of blight-resistant American chestnut trees will undoubtedly be monitored for many attributes. Population genetic concerns will likely center on the presence of founder effects, the effects of inbreeding within populations, and population expansion

(see Section V.B). The outcome of ecological interactions between blight-resistant and native chestnuts, as well as between blight-resistant chestnut trees and the chestnut blight fungus, remains to be seen. Genetic markers located in both the nuclear and the chloroplast genomes of American chestnut can be used to address many questions of genetic diversity, structure, and mating within populations and to paint a picture of how populations expand and interact. Tracking associations among morphology, phenology, genetics, and survival will help breeders and silviculturists to be confident that the right trees are planted at the right location.

1. Chloroplast Markers. Chloroplast DNA (cpDNA) markers located on the maternally inherited chloroplast genome have proven useful in understanding aspects of chestnut population expansion, colonization, and interpopulation interactions where the diversity, evenness, and aggressiveness of maternal lineages is in question (Dane et al. 1999; Lang et al. 2007). A central question in chestnut reintroduction concerns the ability of newly planted populations to colonize new territories by long-distance seed dispersal. It can be inferred that species with broader ranges are potentially good colonists since they managed to spread in the past (Petit et al. 2004). Indeed, broad-ranging species have distinct genetic features that include relatively high gene diversity and less differentiation among populations at isozyme markers than species with narrower ranges (Hamrick, Godt, and Sherman-Broyles 1992). American chestnut expanded its range rapidly after the recession of the Pleistocene glaciers (Delcourt and Delcourt 1984; Huang et al. 1994) and has been shown to outgrow other hardwood species in plantings (Paillet and Rutter 1989; Jacobs and Severeid 2004), findings that further support the high colonizing potential of the species. Because colonization of new territories relies on seed dispersal, the level of differentiation among populations at maternally inherited markers may shed some light on the rate of seed dispersal, the relative dispersal ability of maternal lines, and the diversity of cpDNA haplotypes in newly established colonies. Maternal markers can also be used to study the strength and longevity of patches of family structure that develop as the result of a single seed founding a new colony, where each patch represents a maternal clone of large size. In simulation studies with oaks, for example, patches of tens of square kilometers were found to be nearly fixed for a single cpDNA haplotype (Lecorre et al. 1997).

When multiple introductions are employed, genetic studies of zones of secondary contact (where two reintroduced populations meet) can provide information regarding the mode of expansion of populations,

depending on whether the expanding lineages form abrupt "suture zones," intermingle, or form gradual clines (Hewitt 1988). In ponderosa pine, analysis of maternally inherited markers revealed that populations expanded in a diffuse "moving front," resulting in abrupt contact, whereas paternally inherited cpDNA markers showed a progressive cline (Latta and Mitton 1999; Johansen and Latta 2003).

Chloroplast markers have been employed in several studies of *Castanea* genetic diversity and phylogeny Asia, Europe, and North America (e.g., Lang et al. 2006, 2007). American chestnut cpDNA can be distinguished from that of other chestnut species by a 75 basis point (bp) deletion in the *trn*T-L intergenic spacer region (Kubisiak and Roberds 2003; Lang et al. 2006). Nucleotide variation for noncoding cpDNA intergenic spacer regions is generally low in interspecific comparisons of *Castanea*, and sequence divergence among and within genera is low (Fineschi et al. 2000; Lang et al. 2006). Patterns of the variation in the chloroplast DNA of *C. dentata* and *C. pumila* were geographically structured and not congruent with the current phylogeny based on bur and cupule characteristics, leading the authors to suggest chloroplast capture via introgression in North American *Castanea* species (Lang et al. 2006).

Major challenges remain if chloroplast DNA is to be used to study the reintroduction of blight-resistant American chestnut. Markers would be needed that distinguish maternal lines within a species, and to date no intraspecific variation has been detected within the *trn*T-L-F intergenic regions of the chloroplast genome of American chestnut (Lang et al. 2007). The entire chloroplast genome of *C. sativa* had been sequenced (Sebastiani 2003), leading to the development of chloroplast simple sequence repeats (cpSSRs) (Sebastiani et al. 2004) that could be used in monitoring lineages of American chestnut. The newly developed cpSSRs may reveal polymorphisms necessary for monitoring large numbers of American chestnut lineages; alternatively, falling costs of high-throughput second-generation sequencing technologies offer the possibility of completely sequencing the chloroplast genomes of a sample of individuals for finer-scale monitoring of dispersal patterns, mutations, and introgressions.

2. Nuclear Markers. Nuclear markers have been used to examine genetic diversity, heterozygosity, and population differentiation between founder populations and their descendants and between orchard populations and nearby wild or coppiced populations. Allozyme markers were used to detect a zone of hybridization between two distinct races of *Castanea sativa*, an area of gene pool intergradation that the authors

attribute to secondary contact between races that have been differentiated in allopatry (Villani et al. 1999b). Eriksson et al. (2005) found that gene flow and genetic differentiation among wild and coppiced populations of *C. sativa* were higher than among orchard populations, although that was not the case in Japan, where orchard populations showed similar heterozygosities, allele size ranges, and genetic differentiation as wild populations of *C. crenata* (Tanaka et al. 2005). The incongruence in these results may have to do with orchard establishment: In Europe, allochthanous chestnuts often were introduced into cultivated populations whereas in Japan, cultivars seem to have originated from local gene pools (Tanaka et al. 2005).

Nuclear markers may also reveal zones of population admixture, levels of gene flow, and ranges and variances of effective number of males in reintroduced populations. The careful characterization of Turkish chestnut populations by Villani and colleagues illustrates the variety of ways in which population structure and admixture can be examined using nuclear markers. Populations of *C. sativa* in Turkey span an east-west range characterized by a low-rainfall Mediterranean climate in the west and a much higher rainfall Eurosiberian climate in northeastern Anatolia near the Black Sea. Eastern and western populations were shown to be genetically divergent at allozyme loci and for morphological and physiological traits, with populations of intermediate genetic makeup existing between them that are thought to be the result of introgression (Villani et al. 1991, 1992, 1994). Villani et al. (1999b) used 12 allozyme systems to survey 34 populations of *C. sativa* spanning the eastern, intermediate, and western genetic zones in Turkey, and estimated genetic structure and gene flow both within and between zones. They found that most of the overall genetic variance was due to heterogeneity among populations ($F_{ST} = 0.184$) and that allele frequencies were relatively homogenous across regions eastward and westward of the introgression zone, where a sharp transition in allele frequencies and an increase in genetic variability (spanning a cline of 324 km) was clearly detected. Number of migrants between populations (Nm) decreased with geographic distance; populations less than 30 km apart exchanged between 4 and 32 (median of 12) migrants per generation, whereas Nm flattened out at values of 2 to 4 for populations separated by 60 to 90 km. Interestingly, the researchers found that gene flow was significantly higher within genetic zones than between them and that there was a significant decrease of gene flow at the boundaries among the three groups of populations, forming a "partial genetic barrier" among them (Villani et al. 1999b). The authors suggested that this barrier was caused by a limitation in the number of populations in the region of

introgression that could behave as genetic links between the eastern and western populations.

3. Morphological, Physiological, and Phenological Traits. In addition to molecular markers, the genetic diversity of chestnut can be characterized using a variety of morphological, physiological, and phenological traits. Although variability in vegetative morphological traits may be used to distinguish among races or ecotypes of a species, their connection to ecological processes is not obvious, and for that reason most researchers focus on traits such as bud burst, bud set, and timing of flowering, which are closely associated with adaptation, a topic that will be considered later in this chapter. Flowering phenology—the dates when male and female flowering begins and ends—may be the single most important phenological variable because it affects whether individuals and populations will intermate or not (Slavov et al. 2005).

Villani et al. (1999a) suggested that water use efficiency (WUE) was an adequate physiological indicator of satisfactory growth in water-limited environments. They made controlled crosses of trees within and between two genetically divergent *C. sativa* populations in Turkey, measured 351 seeds for 12 morphometric traits, and measured WUE in a sample of 6-month-old seedlings. The progeny of within-population crosses showed significantly more homogeneity in all variables than the between-population crosses. Diskin and others (2006) characterized American chestnuts, Chinese chestnuts, and four hybrid chestnut types using an index of species identity (ISI) calculated from 26 morphometric variables based on leaf, stipule, twig, and bud traits. Although the traits were not necessarily adaptive, they represented consistent morphological differences between American and Chinese chestnuts, were well described, and baseline data including trait means and standard errors were given for each species and hybrid type. These traits might be useful in determining differentiation between populations and may also reveal differences in the retention of Chinese chestnut genomic regions between populations.

Analysis of morphological traits can be coupled with analysis of neutral genetic variation to determine whether local adaptation has occurred in natural populations, without the need for large and expensive common garden plantings. The proportion of variation between populations at marker loci (F_{ST}) can be compared to Q_{ST}, an analogous measure of differentiation at quantitative loci based on the ratio of between population genetic variance to total genetic variance for the trait under study. If morphological or physiological traits show significantly higher differentiation than neutral markers ($Q_{ST} > F_{ST}$), it can be

concluded that divergent selection is shaping adaptive traits (McKay and Latta 2002).

D. Interactions between Resistant and Native Chestnuts

Some blight-resistant American chestnuts will be reintroduced to areas where chestnut formerly grew or still grows, offering a potential genetic connection between native populations of American chestnuts and reintroduced hybrids. The potential for gene flow out of the introduced populations and into the forest is limited, however, because stump sprouts of autochthonous trees now grow in the forest understory, where conditions of low light inhibit flowering (Griffin 1989). Native trees and their moderately susceptible offspring may flower only rarely or may not flower at all, and they may not cross with the BC_3F_3s in nearby plantations. Gametes from moderately susceptible trees that do flower and flow back into the plantation will be swamped, and any resulting offspring will be, on average, much less viable than offspring that result from intraplantation crosses. Crosses between moderately resistant trees and wild trees will be at a strong selective disadvantage to new moderately resistant seedlings produced by plantation $\times$ wild crosses. Finally, parent-offspring matings between moderately resistant trees and nearby wild trees are expected to result in reduced seed set and/or reduced fitness. Thus, moderately resistant trees may not have many viable options for "rescuing" wild American chestnut genes, and their ability to contribute to the blending of blight-resistant hybrids with wild trees could be minimal unless they are managed in a way that promotes the production of flowers and the survival of moderately resistant offspring.

E. Linkage Disequilibrium

Under the plan outlined by the TACF, the third backcross generation, which will be used to reconstitute a population for reintroduction, will be on average 15/16ths American chestnut and 1/16th Chinese chestnut (Rutter and Burnham 1982; Diskin et al. 2006; Liu and Carlson 2006). If the introgressed portion of the Chinese chestnut genome confers no fitness advantage, chromosomal recombination in subsequent meioses would effectively break up the remaining portions of Chinese genome (Lynch and Walsh 1998). However, when selection favors and rapidly increases the frequency of an advantage-conferring locus, sites linked to the favorable locus hitchhike for the ride (Maynard Smith and Haigh 1974). This phenomenon, often called linkage drag, is commonly

encountered in plant breeding when artificial selection favors an introgressed region and drags along unfavorable or neutral linked genes (Brinkman and Frey 1977). In the same way, when natural selection favors a particular allele, sites linked to that locus are dragged along to fixation, resulting in reduced genetic variation in a region around the gene under selection relative to the rest of the genome, a process known as a selective sweep (Walsh 2008). The more rapid the gene fixation, the more reduced the level of variation around the favored site and the larger the size of the region influenced by the sweep. Likewise, as recombination decreases, the length of the sweep-influenced region increases. Specifically, Kaplan et al. (1989) showed that the approximate distance d at which a neutral site can be influenced by a sweep is a function of the strength of selection s and the recombination fraction c,

$$d \cong 0.01 \frac{s}{c}$$

High effective levels of recombination result in a shorter window of influence around the selective site, resulting in shorter regions of disequilibrium (Walsh 2008). Chromosomal regions with reduced recombination such as those caused by chromosome inversions or translocations in hybrids may lead to the production of large linkage blocks that will resist reduction and potentially contribute to evolutionary change (Hoffman and Rieseberg 2008).

Linkage disequilibrium has important evolutionary consequences, as it decreases levels of genetic variation and therefore decreases the efficiency of selection on linked genes within the region influenced by the sweep. These effects occur because (1) linkage disequilibrium reduces the number of independent loci and introduces a negative component of variation that is subtracted from the total genetic variance (the Bulmer effect; Bulmer 1971; Lynch and Walsh 1998). Furthermore, within the region influenced by the sweep, deleterious alleles have a higher probability of fixation, while favorable alleles have a reduced probability of fixation compared to sites outside of the sweep (Walsh 2008). Once the favored site has become fixed, signal for the sweep starts to decay through recombination and mutation, but linkage disequilibrium generated by a selective sweep may persist for at least N_e generations (Przeworski 2002). Even if the generation time for American chestnut is only 10 years (it is likely to be much longer in the forest) and if the N_e is greater than 500 (which seems probable), the linkage disequilibrium caused by selective sweeps around the loci conferring blight resistance could be expected to last for thousands of years in reintroduced American chestnut populations. Genes from Chinese chestnuts in

linkage blocks associated with resistance to *C. parasitica* can be expected to influence hybrid American chestnut evolution for a very long time.

F. Local Adaptation and Long-Term Fitness of Reintroduced Populations

Populations of forest trees are locally adapted when they display the highest relative fitness in their home environment and lower relative fitness in other environments (Savolainen et al. 2007). At any given location along a species' range, there exists a function that describes the species' fitness at that particular location. The individuals with the optimum phenotype have the highest fitness, and as the phenotype deviates from the optimum, fitness declines. Slatkin (1978) showed that a population's ability to track the phenotypic optimum depends on the variance of gene flow into the population, the strength of selection, and the additive genetic variance of the trait at each location. If dispersal distances are short and selection is considerable, adaptation to a fine-scaled environment is possible (Slatkin 1978). Using a different model of migration, the island model (where each subpopulation is of equal size and receives a constant proportion of migrants from the other populations), LeCorre and Kremer (2003) showed there is a trade-off between differentiation of individual loci and between-population linkage disequilibrium. With strong selection and high gene flow, phenotypic differentiation can occur due to small allelic frequency differences between populations. If there is weak stabilizing selection within populations, there will be a great deal of linkage disequilibrium among populations. Consequently, if linkage disequilibrium among populations is pronounced, a strong selection differential will be required to produce concomitant phenotypic differentiation.

Convincing evidence for local adaptation exists for chestnuts and other forest tree species with similar life history traits. Villani et al. (1999b) determined that European chestnuts occupying strikingly different climactic zones were genetically divergent at allozyme loci and for morphometric and physiological traits. Differences between the populations could be seen when within- and between-population crosses were planted in a common garden (Villani 1999a). Steiner (2006) noted that variation in growth rate of American chestnuts from different provenances (populations from well-defined geographic areas) often appeared random when planted in common gardens, an observation that may indicate that patterns of growth differentiate at the microsite level rather than at the provenance level. Similarly, provenance tests of northern red oak (*Quercus rubra*) showed that most of the variation for

growth rate was found within populations, even when the populations were up to 2000 km apart, and that variation in growth rate among northern red oak populations did not show any clear geographic pattern (reviewed in Steiner 1998).

Although these examples present local adaptation on scales of hundreds of kilometers, Steiner and Berrang (1990) reported microsite adaptation to cold injury in pitch pine (*Pinus rigida*), a species with a distribution similar to American chestnut. Seedlings from pitch pines growing in an area with unusually low nighttime temperatures yearround were grown under controlled conditions with pitch pine seedlings from nearby stands (approximately 8 km away) that experienced more moderate nighttime temperatures. The seedlings from the low-temperature area acclimated more rapidly in the fall, resisted colder temperatures, and resumed growth more slowly in the spring. Gene flow between these sites was highly probable; thus, selection pressure was strong enough to differentiate adaptive traits in populations separated by only a few kilometers. Alternatively, Steiner and Berrang (1990) may have witnessed the epigenetic phenomenon of imprinting, whereby temperatures during embryo development, not the genetics of the individuals, imparted a genetic predisposition to cold-hardiness (Johnsen and Skrøppa 1996).

As a general rule in forest genetics, populations of tree species adapted to cold or dry climates may perform favorably almost anywhere (i.e., in climates that are warmer or colder, wetter or drier) whereas populations from warmer and wetter climates perform poorly when planted in areas colder or drier than their original provenance (White, Adams, and Neale 2007). Local adaptation for timing of spring bud burst, a phenological measurement associated with cold injury, has been observed along latitudinal or altitudinal clines in natural populations of sessile oak (*Quercus petraea*; Ducousso et al. 1996), northern red oak (McGee 1974), and pitch pine (Steiner and Berrang 1990), to name a few. An analysis of several adaptive traits in Douglas-fir (*Pseudotsuga menziesii*), a species often found in mountainous areas, showed complex patterns of variation that were accounted for by a combination of latitude, longitude, altitude, and slope (Rehfeldt 1989). Many more examples of local adaptation in forest trees have been reported; see Savolainen et al. (2007) for a thorough review.

The possible absence of local adaptation is a concern for long-term fitness of reintroduced trees, but the overall size (effective population size, *Ne*) of introduced populations may also limit their ability to adapt. Genetics theory predicts that small populations will show a reduced response to long-term selection because they contain lower levels of variation to begin with, are more susceptible to drift during the selection

process, and produce new mutations less frequently (Hill and Rabash 1986; Willi et al. 2006). Individuals in small populations, moreover, often have low mean fitness due to inbreeding depression, genetic load, or nongenetic Allee effects (Stephens and Sutherland 1999). Low individual fitness, as well as stressful environmental conditions, reduces the growth rate of a population, which in turn compromises its potential response to selection and increases the likelihood of local extinction through stochasticity (Menges 1998; Willi et al. 2006). Finally, if heritability is decreased due to environmental stresses or inbreeding, the population-reducing effects of selection will be further exacerbated by the population's increasing lag behind the optimal phenotype (Lynch and Lande 1993).

Chestnut breeders are using several methods to reduce the possibility of reintroducing maladapted trees into the landscape. The primary method is to include in the breeding program as many locally adapted sources of American chestnut germplasm as possible. Ideally, hybrids will be introduced back into the area from which their American chestnut parent originated. Under the TACF breeding model, each state chapter is striving to find 20 or more American chestnuts to use in the backcross breeding program for that state (Hebard 2005). Resistant selections are expected to be planted and allowed to open pollinate in three regional seed orchards representing the southern, central, and northern areas of the species' range within the United States. If many relatively small populations are introduced (rather than a few large populations), the loss of one population will not have as dramatic an effect on the overall reintroduction effort. A strategy that employs many small reintroductions increases the likelihood that some of the populations either will be on sites to which they are adapted or will be able to disperse to suitable sites.

The TACF breeding program was designed to capture of as much genetic diversity as possible from American chestnuts, with the goal of maximizing local adaptation. It is reasonable to assume that alleles conferring adaptation to a range of environments were present in preblight populations of American chestnut and that high gene flow and high outcrossing rates perpetually created new combinations of alleles that were more favorable in some environments than others. If the TACF breeding program has captured a large portion of existing alleles, then outcrossing within and between reintroduced populations should again lead to genotypes that are locally adapted to specific sites.

Finally, the problem of small populations inhibiting local adaptation can be ameliorated by continual supplementation with new genotypes. Assuming a nonzero survival rate, continual addition of new individuals

should ensure that at least some trees will survive to adulthood and reproduce (Ryman and Laikre 1991). One possible scheme for reintroduction that lends itself to population supplementation is the reintroduction of populations in cycles. According to this scheme, populations are reintroduced at specific geographic points such that gene flow could occur between them. Some years later, more populations are introduced to fill the gaps between existing populations and to add more alleles to the total population. In this model, cost and planting and management effort are spread out over a number of years, and improvements in the TACF breeding population (i.e., more sources of resistance and more American chestnut genes) have a well-defined mechanism to enter the landscape.

G. Lessons from Other Reintroduction Programs

American chestnut formerly occupied over 800,000 km^2 of the eastern United States (Braun 1950), and chestnut breeders face unique challenges in attempting to reintroduce American chestnut hybrids into the entire former range. Conservation breeders who work with plants can learn from the successes and failures of the most sophisticated and advanced examples of captive breeding for release into the wild: the salmonid breeding programs of the northern Pacific Rim (Fraser 2008). Frankham (2008) suggested that the larger the effective population size (N_e) of a breeding population is, the more successful that breeding program will be at maintaining genetic diversity. Methods that increase N_e in a breeding population may include the maintenance of a high census population (N), starting the breeding population with as many genetically diverse founders as possible, and minimizing the family variance of reintroduced populations (Ryman and Laikre 1991) by equalizing family sizes in the final stage of the breeding program. Releasing individuals into the wild at the proper stage of growth, at the proper time of year, and into a properly prepared environment improves the survival chances upon exposure to the new environment. Finally, monitoring the success of captive-bred individuals is crucial to receiving feedback necessary for improvement of a captive-breeding program (Fraser 2008).

VI. CONCLUSION

Based on the preceding review and lessons from other captive breeding programs, a number of general conclusions regarding chestnut

reintroduction can be made. It will be critical that the effective population size of the breeding population be maintained at a high level by incorporating as many American chestnut alleles as possible and by managing breeding populations such that equal variance in family sizes characterizes the final generation for reintroduction. Many locally adapted genotypes should be included in the program, and genotypes should be deployed to their provenance of origin to preserve both genetic diversity and local adaptation. In order to increase chances of seed germination and seedling survival, factors that favor local regeneration should be identified and sites for reintroduction prepared accordingly. This may involve determining the best types of planting stock, simulating disturbance, controlling competition, and/or protecting reintroduced populations from herbivory. As many unrelated individuals as possible should be deployed in small populations such that within- and between-population matings are possible. Reintroduction sites should be managed and the long-term progress of the trees monitored. Monitoring may include measuring survival, fecundity, variance in reproductive success, the retention of Chinese chestnut genome, the extent of gene flow into and out of introduced populations, and any changes in growth or development that may indicate inbreeding depression. Special attention should be given to population genetic parameters and the fitness of the first generation of trees that regenerates following introduction, as these trees will provide information that will characterize subsequent generations. To close the loop, chestnut breeders must incorporate lessons learned from early reintroductions into future breeding and deployment decisions. The plans of the TACF already embody many of these suggestions.

An important distinction between the salmonid breeding programs and the work of the TACF is that salmon were threatened primarily by loss of habitat, whereas chestnut is threatened primarily by exotic disease. Incorporating as many sources of blight resistance into reintroduced populations will help increase the variability of resistance and protect the trees from genetic changes in the pathogen. Because interactions between a plant host with a hybrid genome and a fungal pathogen are complex and difficult to predict, reintroduced American chestnut populations will require careful monitoring for incidence of chestnut blight. In addition, attacks by other known pests and pathogens of chestnut, such as root rot fungi (*Phytophthera* spp.) and chestnut gall wasp (*Dryocosmus kuriphilus*), are certain. The threat posed by gypsy moth (*Lymantria dispar)*, twolined chestnut borer (*Agrilus bilineatus*), ambrosia beetles (Curculionidae), and other potential chestnut pests and pathogens remains to be seen.

Social forces—such as the public's acceptance of new trees, their perception of the reintroduction, and their feelings about changes in the forest as a result of the reintroduction—will influence when and where reintroduced American chestnut populations will be successful. While these social forces, as well as the biotic and abiotic considerations enumerated earlier, are challenges that can be planned for and met, they also represent a large area of uncertainty that will individually or collectively have a large effect on where American chestnut hybrids will grow in the future. American chestnut will be the first major tree species to be reintroduced throughout its former range (or, to be exact, to have its populations significantly supplemented) by a conservation breeding program. Chestnut could set the stage for the reintroduction or supplementation of other tree species facing similar threats, including hemlock (*Tsuga canadensis*) and butternut (*Juglans cinera*).

LITERATURE CITED

Alexander, M.T., L.M. Worthen, and J.H. Craddock. 2005. Conservation of *Castanea dentata* germplasm in the southeastern United States. Acta Hort. 693:485–490.

Allee, W.C. 1938. The social life of animals. Norton, New York.

Anagnostakis, S.L., B. Hau, and J. Kranz. 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. Plant Dis. 70:536–538.

Anagnostakis, S.L. 1987. Chestnut blight—the classical problem of an introduced pathogen. Mycologia 79(1):23–37.

Anagnostakis, S.L. 1992. Chestnuts and the introduction of chestnut blight. Annu. Rep. Northern Nut Growers Assoc. 83:23–37.

Austerlitz, F., S. Mariette, N. Machon, P.H. Gouyon, and B. Godelle. 2000. Effects of colonization processes on genetic diversity: differences between annual plants and tree species. Genetics 154:1309–1321.

Berg, E.E., and J.L. Hamrick. 1995. Fine-scale genetic structure of a turkey oak forest. Evolution 49:110–120.

Braun, E. 1950. Deciduous forests of eastern North America. Macmillan, New York.

Bossema, I. 1979. Jays and oaks: and eco-ethological study of a symbiosis. Behaviour 70:1–117.

Brewbaker, J.L. 1957. Pollen cytology and self-incompatibility in plants. J. Hered. 48:271–277.

Brinkman, M.A., and K.J. Frey. 1977. Yield component analysis of oat isolines that produce different grain yields. Crop Sci. 17:165–168.

Buiteveld, J., E. G. Bakker, J. Bovenschen, and S.M.G. deVries. 2001. Paternity analysis in a seed orchard of *Quercus robur* L. and estimation of the amount of background pollination using microsatellite markers. Forest Gen. 8(4):331–337.

Bulmer, M.G. 1971. The effect of selection on genetic variability. Am Nat 105:201–211.

Burnham, C.R. 1982. Breeding for chestnut blight resistance. Nutshell 35:8–9.

Burnham, C.R. 1986. Chestnut hybrids from USDA breeding programs. J. Am. Chestnut Found. 1(2):8–12.

Burnham, C.R., P.A Rutter, and D.W. French. 1986. Breeding blight-resistant chestnuts. Plant Breed Rev. 4:347–397.

Buttrick, P.L. 1925. Chestnut in North Carolina. pp. 6–10. In: Chestnut and the chestnut blight in North Carolina. North Carolina Geology and Economy Paper 56.
Byers, D.L., and T.R. Meagher. 1992. Mate availability in small populations of plant species with homomorphic sporophytic self-incompatibility. Heredity 68:353–359.
Casasoli, M., C. Mattioni, M. Cherubini, and F. Villani. 2001. A genetic linkage map of European chestnut (Castanea sativa Mill.) based on RAPD, ISSR, and isozyme markers. Theor. Appl. Genet. 102:1190–1199.
Clapper, R.B. 1952. Relative blight resistance of some chestnut species and hybrids. J Forestry 50:453–455.
Clapper, R.B. 1963. A promising new forest-type chestnut tree. J. For. 61:921–922.
Clark, J.S., C. Fastie, G. Hurtt, S.T. Jackson, C. Johnson, G.A. King, M. Lewis, J. Lynch, S. Pacala, C. Prentice, E.W. Schupp, T. Webb III, and P. Wyckoff. 1998. The Reid's paradox of rapid plant migration: Dispersal theory and interpretation of paleoecological records. Bioscience 48:13–27.
Cook, R., and H.S. Forest. 1979. The American chestnut II: Chestnuts in the Gennessee Valley region, 1978. The Rochester Committee for Scientific Inform. Bul. 226:1–9.
Cousens, R., C. Dytham, and R. Law. 2008. Dispersal in plants: A population perspective. Oxford Univ. Press, New York.
Cunningham, I.S. 1984. Frank N. Meyer, plant hunter in Asia. Iowa State Univ. Press, Ames.
Dane, F., L. K. Hawkins, and H. Huang. 1999. Genetic variation and population structure of *Castanea pumila* var. *ozarkensis*. J. Am. Soc. Hort. Sci. 124:666–670.
Dane, F., P. Lang, H. Huang, and Y. Fu. 2003. Intercontinental genetic divergence of *Castanea* species in eastern Asia and eastern North America. Heredity 91:314–321.
Daubree, J.B., and A. Kremer. 1993. Genetic and phenological differentiation between introduced and natural populations of *Quercus rubra* L. Ann. Sci. Forestry 50 (Suppl. 1):271s–280s.
Delcourt, H.R. 2002. Forests in peril: Tracking deciduous trees from ice-age refuges into the greenhouse world. McDonald and Woodward Publ., Granville, OH.
Delcourt, H.R., and P.A. Delcourt. 1984. Ice age haven for hardwoods. Nat. Hist. 93:22–28.
Detwiler, S.B. 1915. The American chestnut tree. Am. Forester 21:957–960.
Diller, J.D., and R.B. Clapper. 1969. Asiatic and hybrid chestnut trees in the eastern U.S. States. J. Forestry 67:328–331.
Diskin, M., K.C. Steiner, and F.V. Hebard. 2006. Recovery of American chestnut characteristics following hybridization and backcross breeding to restore blight ravaged *Castanea dentata*. Forest Ecol. Manag. 223:439–447.
Double, M.L., and W.L. Macdonald. 2002. Hypovirus deployment, establishment, and spread: Results after six years of canker treatment. Phytopathology 92:S94.
Dow, B.D., and M.V. Ashley. 1998. High levels of gene flow in bur oak revealed by paternity analysis using microsatellites. J. Hered. 89:62–70.
Ducousso, A., J.P. Guyon, and A. Kremer. 1996. Latitudinal and altitudinal variation of bud burst in western populations of sessile oak (*Quercus petraea* (Matt) Liebl). Ann. Sci. Forestry 53:775–782.
Ellstrand, N.C., and D.R. Elam. 1993. Population genetic consequences of small population size: Implications for plant conservation. Annu Rev. Ecol. Evol. Syst. 24:217–242.
Eriksson, G., A. Pliura, J. Fernandez-Lopez, R. Zas, R. Blanco-Silva, F. Villani, G. Bucci, M. Casasoli, M. Cherubini, M. Lauteri, C. Mattioni, C. Monteverdi, A. Sansotta, G. Garrod, M. Mavrogiannis, R. Scarpa, F. Spalato, P. Aravanoupoulos, E. Alizoti, A. Drouzas, C. Robin, T. Barreneche, A. Kremer, R. Botta, D. Marinoni, and A. Akkak. 2005. Management of genetic resources of the multi-purpose tree species *Castanea sativa* Mill. Acta. Hort. 693:373–386.

Falconer, D.S. 1960. Introduction to quantitative genetics. Ronald, New York.
Falconer, D.S., and T.F.C. Mackay. 1996. Introduction to quantitative genetics, 4th ed. Longman, Essex, UK.
Fernandez-Lopez, J., and R. Alia. 2000. Chestnut (*Castanea sativa*) genetic resources conservation strategy. Intl Plant Genetic Res. Inst. http://www.ipgri.org. (Accessed July 17, 2008.)
Fineschi, S., D. Taurchini, F. Villani, and G.G. Vendramin. 2000. Chloroplast DNA polymorphism reveals little geographical structure in *Castanea sativa* Mill. (Fagaceae) throughout southern European countries. Mol. Ecol. 9:1495–1503.
Frankham, R. 2008. Genetic adaptation to captivity. Mol. Eco. 17:325–333.
Fraser, D.J. 2008. How well can captive breeding programs conserve biodiversity? A review of salmonids. Evol. Applications 1:535–586.
Grente, J., and S. Sauret. 1969. L'hypovirulence exclusive, phénomène original en pathologie végétale. C.R. Acad. Sci. 268:3173–3176
Grente J., and S. Berthelay-Sauret. 1978. Biological control of chestnut blight in France. pp. 4–5. In: W.L. MacDonald, F.C. Chech, J. Luchok, and H.C. Smith (eds.), Proceedings of the American Chestnut Symposium. West Virginia Univ. Books, Morgantown.
Griffin, G.J. 2000. Blight control and restoration of the American chestnut. J. Forestry 98 (2):22–27.
Griffin, G.J. 1989. Incidence of chestnut blight and survival of American chestnut in forest clearcut and neighboring understory sites. Plant Dis. 73:123–127.
Griffin, G.J., F.V. Hebard, R.W. Wendt, and J.R. Elkins. 1983. Survival of American chestnut trees: Evaluation of blight resistance and virulence in *Endothia parasitica*. Phytopathology 73:1084–1092.
Grubb, P.J. 1977. The maintenance of species-richness in plant communities: the importance of the regeneration niche. Biol. Rev. 52:107–145.
Hamrick, J.L., M.J.W. Godt, and S.L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. New Forests 6:95–124.
Han, J.C., G.P Wang, D.J. Kong, Q.X. Liu, and X.Y. Zhang. 2007. Genetic diversity of Chinese chestnut (*Castanea mollissima*) in Hebei. Acta Hort. 760:573–577.
Hebard, F.V. 1994. The American Chestnut Foundation breeding plan: beginning and intermediate steps. J. Am. Chestnut Found. 8:21–28.
Hebard, F.V. 2002. Notes from Meadowview Research Farms 2001–2002. J. Am. Chestnut Found. 16:7–18.
Hebard, F.V. 2004. Notes from Meadowview Research Farms 2003–2004. J. Am. Chestnut Found. 18:34–35.
Hebard, F.V. 2005. Notes from Meadowview Research Farms 2004–2005. J. Am. Chestnut Found. 19:27–39.
Hebard, F.V. 2006. The backcross breeding program of the American Chestnut Foundation. pp. 61–77. In: K.C. Steiner, and J.E. Carlson (eds.), Restoration of the American chestnut tree to forest lands —Proceedings of a conference and workshop. May 4–6, 2004, North Carolina Arboretum. Natural Resources Rep. NPS/NCR/CUE/NRR—2006/001, National Park Service, Washington, DC.
Hewitt, G.M. 1988. Hybrid zones—natural laboratories for evolutionary studies. Trends Ecol. Evol. 3:158–167.
Hill, W.G., and J. Rabash. 1986. Models of long-term artificial selection in finite populations with recurrent mutation. Genet. Res. 48:125–131.
Hoffman, A.A., and L.H. Rieseberg. 2008. Revising the impact of inversions in evolution: From population genetic markers to drivers of adaptive shifts and speciation? Annu. Rev. Ecol. Evol. Syst. 39:21–42.

Huang, H., W.A. Carey, F. Dane, and J.D. Norton. 1996. Evaluation of Chinese chestnut cultivars for resistance to *Cryphonectria parasitica*. Plant Dis. 80:45–47.
Huang, H., F. Dane, and T.L. Kubisiak. 1998. Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut (Fagaceae). Am. J. Bot. 85:1013–1021.
Huang, H., F. Dane, and J.D. Norton. 1994. Allozyme diversity in Chinese, Seguin, and American chestnut (*Castanea* spp.). Theor. Appl. Genet. 88:981–985.
Illick, J. 1928. Pennsylvania trees. Pennsylvania Dept. Forest and Water Res. Bul. 11. Harrisburg, PA.
Irwin, H. 2003. The road to American chestnut restoration. J. Am. Chestnut Found. 16 (2):6–13.
Jacobs, D.F. 2007. Toward development of silvical strategies for forest restoration of American chestnut (*Castanea dentata*) using blight-resistant hybrids. Biol. Conserv. 137:497–506.
Jacobs, D.F., A. Ross-Davis, and A.S. Davis. 2004. Establishment success of conservation tree plantations in relation to silvicultural practices in Indiana, USA. New Forest 28:23–36.
Jacobs, D.F., and L.R. Severeid. 2004. Dominance of interplanted American chestnut (*Castanea dentata*) in southwestern Wisconsin, USA. Forest Ecol. Managm. 191:111–120.
Jaynes, R.A. 1974. Genetics of chestnut. Forest Service, USDA, Washington, DC.
Jaynes, R.A. 1975. Chestnut. pp. 590–503. In: J. Janick and J. Moore (eds.), Advances in fruit breeding. Purdue Univ. Press, West Lafayette, IN.
Jaynes, R.A. 1978. Selecting and breeding blight resistant chestnut trees. pp. 4–6. In: W.L. MacDonald, F.C. Cech, J. Luchok, and C. Smith (eds.), Proceedings of the American chestnut symposium. West Virginia Univ. Books, Morgantown, WV.
Jensen, T.S., and O.F. Nielsen. 1986. Rodents as seed dispersers in a heath-oak wood succession. Oecologia 70:214–221.
Johansen, A.D., and R.T. Latta. 2003. Mitochondrial haplotype, distribution, seed dispersal, and patterns of postglacial expansion of ponderosa pine. Molec. Ecol. 12:293–298.
Johnsen, Ø., and T. Skrøppa. 1996. Adaptive properties of *Picea abies* progenies are influenced by environmental signals during sexual reproduction. Euphytica 92:67–71.
Johnson, W.C., and T. Webb. 1989. The role of blue jays (*Cyanocitta cristata*) in the postglacial dispersal of Fagaceous trees in eastern North America. J. Biogeogr. 16:561–571.
Kaplan. N.L., R.R. Hudson, and C.H. Langely. 1989. The "hitchhiking effect" revisited. Genetics 123:887–889.
Kimura, M. 1955. Random genetic drift in multi-allelic locus. Evolution 9:419–435.
Koenig, W., and M.V. Ashley. 2003. Is pollen limited? The answer is blowin' in the wind. Trends Ecol. Evol. 18:157–159.
Kubisiak, T.L., F.V. Hebard, C.D. Nelson, J. Zhang, R. Bernatzky, H. Huang, S.L. Anagnostakis, and R.L. Doudrick. 1997. Molecular mapping of resistance to blight in an interspecific cross in the genus *Castanea*. Phytopathology 87:751–759.
Kubisiak, T.L., and J.H. Roberds. 2003. Genetic variation in natural populations of American chestnut. J. Am. Chestnut Found. 16(2):42–48.
Lang, P., F. Dane, and T.L. Kubisiak. 2006. Phylogeny of *Castanea* (Fagaceae) based on chloroplast *trn*T-L-F sequence data. Tree Genet. Genome. 2:132–139.
Lang, P., F. Dane, T.L. Kubisiak, and H. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. Mol. Phylogenet. Evol. 43:49–59.
Lang, P., and H. Huang. 1999. Genetic diversity and geographic variation in natural populations of the endemic *Castanea* species in China. Acta Bot. Sin. 41:651–657.

Latta, R.G., and J.B. Mitton. 1999. Historical separation and present gene flow through a zone of secondary contact in ponderosa pine. Evolution 53:769–776.

LeCorre, V., and A. Kremer. 2003. Genetic variability at neutral markers, quantitative trait loci, and trait in a subdivided population under selection. Genetics 164:1205–1219.

LeCorre, V., N. Machon, R.J. Petit, and A. Kremer. 1997. Colonization with long-distance seed dispersal and distribution of maternally inherited diversity in forest trees: a simulation study. Genet. Res. 69:117–125.

Lexer, C., D.M. Rosenthal, O. Raymond, L.A. Donovan, and L.H. Rieseberg. 2005. Genetics of species differences in the wild annual sunflowers, *Helianthus annuus* and *H. petiolaris*. Genetics 169:2225–2239.

Liu, S., and J.E. Carlson. 2006. Selection for Chinese vs. American genetic material in blight resistant backcross progeny using genomic DNA. pp. 133–150. In: K.C. Steiner and J.E. Carlson (eds.), Restoration of the American chestnut tree to forest lands—Proceedings of a conference and workshop. May 4–6, 2004, North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR—2006/001, National Park Service, Washington, DC.

Lynch, M., and R. Lande. 1993. Evolution and extinction in response to environmental change. pp. 234–250. In: P.M. Kareiva, J.G. Kingsolver, and R.B. Huey (eds.), Biotic interactions and global change, Sinauer, Sunderland, MA.

Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer, Sunderland, MA.

Maynard Smith, J., and J. Haigh. 1974. The hitch-hiking effect of a favorable gene. Genet. Res. 23:23–35.

McCament, C.L., and B.C. McCarthy. 2005. Two-year response of American chestnut (*Castanea dentata*) seedlings to shelterwood harvesting and fire in a mixed-oak forest ecosystem. Can. J. Forest Res. 35:740–749.

McGee, C.E. 1974. Elevation of seed sources and planting sites affects phenology and development of red oak seedlings. For. Sci. 20:160–164.

McKay, H.K., and R.G, Latta. 2002. Adaptive population divergence: markers, QTL, and traits. Trends Ecol. Evol. 17:285–291.

McKay, J.W. 1942. Self-sterility in the Chinese chestnut (*Castanea mollissima*). Proc. Am. Soc. Hort. Sci. 41:156–160.

McKay, J.W., and H.L. Crane. 1939. The immediate effect of pollen on the fruit of the chestnut. Proc. Am. Soc. Hort. Sci. 36:293–298.

Menges, E.S. 1998. Evaluating extinction risks in plant populations. pp. 49–65 In: P.L. Fielder and P.M. Kareiva (eds.), Conservation biology for the coming decade, 2nd ed. Springer, New York.

Merkel, H.W. 1906. A deadly fungus on the American chestnut. Annu. Rep. New York Zool. Soc. 10:97–103.

Milgroom, M.G., and P. Cortesi. 2004. Biological control of chestnut blight with hypovirulence: A critical analysis. Annu. Rev. Phytopathol. 42:311–338.

Nei, M., T. Maruyama, and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. Evolution 29:1–10.

Newhouse, J.R. 1990. Chestnut blight. Sci. Am. 263(1):106–111.

Paillet, F. L., and P.A. Rutter. 1989. Replacement of native oak and hickory tree species by the introduced American chestnut (*Castanea dentata*) in southwestern Wisconsin. Can. J. Bot. 67:3457–3469.

Petit, R.J. 2004. Biological invasions at the gene level. Diversity Distrib. 10:159–165.

Petit, R.J., and A. Hampe. 2006. Some evolutionary consequences of being a tree. Annu Rev. Ecol. Evol. Syst. 37:187–214.

Petit, R.J., R. Bialozyt, S. Brewer, R. Cheddadi, and B. Comps. 2001. From spatial patterns of genetic diversity to postglacial migration processes in forest trees. pp. 295–318. In: J. Silvertown and J. Antonovics (eds.), Integrating ecology and evolution in a spatial context. Blackwell Science, Oxford.

Petit, R.J., R. Bialozyt, P. Garnier-Gere, and A. Hampe. 2004. Ecology and genetics of tree invasions: From recent introductions to Quaternary migrations. Forest Ecol. Managem. 197:117–137.

Pierson, S.A.M., Keiffer, C.H., McCarthy, B.C., and Rogstad, S.H. 2007. Limited reintroduction does not always lead to rapid loss of genetic diversity: An example from the American chestnut (*Castanea dentata*; Fagaceae). Restor. Ecol. 15:420–429.

Pigliucci, M., C. Paoletti, S. Fineschi, and M.E. Malvolti. 1991. Phenotypic integration in chestnut (*Castanea sativa* Mill.): Leaves versus fruits. Bot. Gaz. (Chicago) 152:514–521.

Poorter, L. 2007. Are species adapted to their regeneration niche, adult niche, or both? Am. Nat. 169(4):433–442.

Przeworski, M. 2002. The signature of positive selection at randomly chosen loci. Genetics 160:1179–1189.

Rajora, O.P., and A. Mosseler. 2001. Challenges and opportunities for conservation of forest genetic resources. Euphytica 118:197–212.

Rehfeldt, G.E., C.C. Ying, D.L. Spittlehouse, and D.A. Hamilton. 1999. Genetic responses to climate change in *Pinus contorta*: Niche breadth, climate change, and reforestation. Ecol. Monogr. 69:375–407.

Richardson, D.M. 1998. Forest trees as invasive aliens. Conserv. Biol. 12:18–26.

Roane, M.K., G.J. Griffin, and J.R. Elkins. 1986. Chestnut blight, other *Endothia* diseases, and the genus *Endothia*. APS Press, St. Paul, MN.

Russell, E.W.B. 1987. Pre-blight distribution of *Castanea dentata* (Marsh.) Borkh. B. Torrey Bot. Club 114:183–190.

Rutter, P.J., and R.B. Burnham. 1982. The Minnesota chestnut program—new promise for breeding a blight-resistant American chestnut. pp. 81–90. 73rd Annu. Rep., Northern Nut Growers Assoc. State University of New York, Geneseo, NY.

Ryman, N., and L. Laikre. 1991. Effects of supportive breeding on the genetically effective population size. Conserv. Biol. 5:325–329.

Savolainen, O., T. Pyhajarvi, and T. Knurr. 2007. Gene flow and local adaptation in trees. Annu. Rev. Ecol. Evol. Syst. 38:595–619.

Sax, D.F., and J.H. Brown. 2000. The paradox of invasion. Global Ecol. Biogeogr. 9:363–371.

Sebastiani, F. 2003. Sequenziamento del genoma del cloroplasto del castagno, *Castanea sativa Mill*. PhD Thesis, Univ. Florence, Italy.

Sebastiani, F., S. Carnevale, and G.G. Vendramin. 2004. A new set of mono- and dinucleotide chloroplast microsatellite markers in Fagaceae. Mol. Ecol. Notes 4:259–261.

Selig, M.F., J.R. Seifert, and D.F. Jacobs. 2005. Response of American chestnut to weed control treatments at plantation establishment. J. Am. Chestnut Found. 19(1):33–41.

Sisco, P.H. 2004. Breeding blight resistant American chestnut trees. J. Am. Chestnut Found. 18(1):15.

Sisco, P.H., T.L. Kubisiak, M. Casasoli, T. Barreneche, A. Kremer, C. Clark, R.R. Sederoff, F.V. Hebard, and F. Villani. 2005. An improved genetic map for *Castanea mollissima*/*Castanea dentata* and its relationship to the genetic map of *Castanea sativa*. Acta Hort. 693:491–495.

Slatkin, M. 1978. Spatial patterns in the distributions of polygenic traits. J. Theor. Biol. 70:213–228.

Slavov, G.T., Howe, G.T., and W.T. Adams. 2005. Pollen contamination and mating patterns in a Douglas-fir seed orchard as measured by simple sequence repeat markers. Can. J. Forestry Res. 35:1592–1603.

Steele, M.A., B.C. McCarthy, and C.H. Keefer. 2005. Seed dispersal, seed predation, and the American chestnut. J. Am. Chestnut Found. 19:47–54.
Steiner, K.C. 1998. A decline-model interpretation of genetic and habitat structure in oak populations and its implications for silviculture. Eur. J. For. Path. 28:113–120.
Steiner, K.C. 2006. Regional adaptation in American chestnut. pp. 123–126. In: K.C. Steiner and J.E. Carlson (eds.), Restoration of the American chestnut tree to forest lands—Proceedings of a conference and workshop. May 4–6, 2004, North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR–2006/001, National Park Service, Washington, DC.
Steiner, K.C., and P.C. Berrang. 1990. Microgeographic adaptation to temperature in pitch pine progenies. Am. Midl. Nat. 123:292–300.
Steiner, K., A. Ellingboe, S. Friedman, F. Hebard, H. Irwin, P. Sisco, and S. Schlarbaum. 2004. TACF adopts guidelines for testing blight-resistant American chestnuts. J. Am. Chestnut Found. 18(1):7.
Stephens, P.A., and W.J. Sutherland. 1999. Consequences of the Allee effect for behavior, ecology, and conservation. Trends Ecol. Evol. 14:401–405.
Stilwell, K.L., H.M. Wilbur, C.R. Werth, and D.R. Taylor. 2003. Heterozygote advantage in the American chestnut, *Castanea dentata* (Fagaceae). Am. J. Bot. 90:207–213.
Stout, A.B. 1928. Dichogamy in flowering plants. B. Torrey Bot. Club 55:141–153.
Streiff, R., T. Labbe, R. Bacilieri, H. Steinkellner, J. Glossl, and A. Kremer. 1998. Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. assessed with isozymes and microsatellites. Mol. Ecol. 7:317–328.
Tanaka, T., T. Yamamoto, and M. Suzuki. 2005. Genetic diversity of *Castanea crenata* in Northern Japan assessed by SSR markers. Breed. Sci 55:271–277.
Vermeij, G.J. 1996. An agenda for invasion biology. Biol. Conserv. 78:3–9.
Villani, F., M. Lauteri, A. Sansotta, and M. Kucuk. 1999a. Genetic structure and quantitative trait variation in F1 full-sib progenies of *Castanea sativa* Mill. Acta. Hort. 494:395–405.
Villani, F., M. Pigliucci, S. Benedettelli, and M. Cherubina. 1991. Genetic differentiation among Turkish chestnut (*Castanea sativa* Mill.) populations. Heredity 66:131–136.
Villani, F., M. Pigliucci, and M. Cherubini. 1994. Evolution of *Castanea sativa* Mill. in Turkey and Europe. Genet. Res. Camb. 63:109–116.
Villani, F., M. Pigliucci, M. Lauteri, and M. Cherubini. 1992. Congruence between genetic, morphometric, and physiological data on differentiation of Turkish chestnut (*Castanea sativa*). Genome 35:251–256.
Villani, F., A. Sansotta, M. Cherubini, D. Cesaroni, and V. Sbordoni. 1999b. Genetic structure of natural populations of *Castanea sativa* in Turkey: Evidence of a hybrid zone. J. Evol. Biol. 12:233–244.
Walsh, B. 2008. Using molecular markers for detecting domestication, improvement, and adaptation genes. Euphytica 161:1–17.
Wang, Y., M. Kang., and H. Huang. 2006. Subpopulation genetic structure in a panmicitc population as revealed by molecular markers: A case study of *Castanea seguinii* using SSR markers. J. Plant Ecol. 30:147–156.
Weeks, S., H. Weeks, and G.R. Parker. 2005. Native trees of the Midwest: Identification, wildlife values, and landscape use. Purdue Univ. Press, West Lafayette, IN.
White, T.L., W.T. Adams, and D.B. Neale. 2007. Forest genetics. CAB Intl, Cambridge, UK.
Willi, Y., J. Van Buskirk, and A.A. Hoffman. 2006. Limits to the adaptive potential of small populations. Annu. Rev. Ecol. Evol. S 37:433–458.
Wright, S. 1931. Evolution in Mendelian populations. Genetics 16:97–159.