MICROSATELLITE GENETIC DIVERSITY OF NORTHERN RED OAK (*QUERCUS RUBRA* L.) IN WESTERN NORTH CAROLINA PRE- AND POST-CHESTNUT BLIGHT AND PRE- AND POST-HARVEST

By

Stephanie Quinet Grant

A Thesis
Submitted to the
Faculty of the Graduate School
In Partial Fulfillment of
the Requirements for the Degree
of
Master of Science

Committee:

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ABSTRACT

The impact of harvest and the loss of the American chestnut (*Castanea dentata* (Marsh)Borkh) was examined in northern red oak (*Quercus rubra* L.) (NRO) using nine microsatellite markers. Four sites located in the Nantahala National Forest in western North Carolina were considered. Each site contained a stand that had not been harvested since the removal of American chestnut circa 1920 and a stand with a more recent harvest history. Comparisons between individuals present in the stands prior to the harvest and individuals regenerated in the stand after the harvest as well as in unharvested stands between pre- and post-chestnut (*Cryphonectria parasitica* (Murill) Barr) blight populations indicated no significant differences in genetic diversity measures including total number of alleles (A_T), mean number of alleles per locus (A), number of private alleles, observed heterozygosity (H_o), expected heterozygosity (H_e), latent genetic potential (LGP) and F_{ST}. Lack of genetic difference between pre- and post- harvest populations suggest NRO populations are either plastic, adapted to micro-climate changes such as those occurring as a result of a harvest, or have already undergone genetic change after the loss of the American chestnut. Significant differences were found between pre- and post- blight populations in terms of effective number of alleles (A_e), genic differentiation and specific allelic frequencies. This suggests that selective pressures in the sampled sites have been altered enough over time to precipitate some genetic change. Ecological factors that have changed in the stands since the loss of the American chestnut include reduced light availability due to change in, gap dynamics, understory species assemblages, and disturbance regime. Gap dynamics have changed

post-chestnut blight in that no trees currently extant in southern Appalachian forests attain the size and therefore leave a comparable sized canopy gap as those left by the American chestnut. Understory species assemblages have changed to include a higher percentage of red maple (*Acer rubrum* L.), a shade tolerant species. In all stands a deficiency of heterozygotes and elevated F_{IS} levels were detected and indicate inbreeding in the stands due to possible restricted pollen flow or habitat fragmentation. In all stands the difference in H_o versus H_e was greater in pre-blight or pre-harvest stands compared to post-blight or post-harvest stands. This may be attributable to change in wind movement and the resulting changes in pollen dispersal across a disturbed landscape.

CHAPTER ONE: INTRODUCTION

Genetic diversity is important to sustainable forestry (Buchert et al., 1997; Riggs, 1990) because it helps provide resistance to factors such as climate change and pest invasion, it promotes stable ecosystems, and has value for artificial breeding (Ledig, 1986). The first step of effective genetic management of a species or population is an assessment of the amount and distribution of genetic variation including compositional, structural and functional components. Genetic composition is the collection of alleles and genotypes present in a population (Griffiths et al., 2008). It is evaluated by the number and type of alleles, and karyotypic variants present. Genetic function includes processes related to fitness such as gene flow, inbreeding and outbreeding (Geber and Griffen, 2003). Genetic structure is the arrangement or distribution of alleles within and among individuals and populations, including patterns of heterozygosity. It is described by comparing individuals within and among populations, but it can also be evaluated across generations in the same population (Geber and Griffen, 2003). Genetic structure is created randomly by drift, or through differential selection of phenotypes resulting in greater survival and reproduction of individuals possessing a certain assemblage of alleles over others (Hartl, 2000). Variation in selective pressures across the geographic range of a species or at smaller scales among populations results in different alleles becoming locally more or less common and creates patterns of spatial or temporal variation in the distribution of alleles (Griffiths et al., 2008).

Genetic diversity initially arises from mutations within individuals, but changes in genetic diversity within populations are also due to processes such as recombination, migration, natural selection and random genetic drift (Hartl, 2000). These processes can

be affected over time by a variety of natural or anthropogenic factors that alter habitat conditions (Adams, 1998; Schagberg et al., 2008). For example, forest management practices that change stand structure and species assemblages can alter understory light conditions (Murcia, 1995) and soil properties (Peng and Thomas 2006; Stoffel et al. 2010; Concilio et al., 2005; Schilling et al. 1999). Forest management can also lead to increased presence of exotics that change competitive relationships (Lindenmayer and McCarthy, 2002; Schilling et al., 1999;Vavra et al., 2007; Anagnostakis, 1987) and introduction of less locally adapted genetic material can occur through artificial regeneration (Adams, 1998; Schagberg et al., 2008). As a result, selection pressures on locally adapted gene complexes can change, thus affecting the relative fitness of individuals, and thus inter- and intra-specific interactions (Atkins and Travis, 2010; Kitzmiller, 1990). Other human activities that lead to habitat fragmentation (Ehrlich and Ehrlich, 1981; Solé et al., 2004), pollutants (Cohen et al. 1993; Terlizzi et al., 2005), loss of habitat (Tillman et al., 2002), and changes in the natural disturbance regimes of ecosystems (Folke et al., 2004) have also been shown to change the dynamics of natural communities, reduce biodiversity and potentially change selection pressures acting on populations. If some members in a population possess alleles that allow them to adaptively respond to these environmental changes more effectively than others in a way that increases their relative fitness, selection may change allele frequencies in the population.

In general, the more alleles present in a population, the higher the likelihood that a portion of those alleles will be associated with a phenotype that allows some individuals in the population to survive despite changes in habitat (Hanksi, 2001). Therefore, the

higher the amount of genetic diversity conserved within species or populations, the greater the potential to adaptively respond to changes in the environment and maintain a viable population size (Falk and Holsinger, 1991). The relatively rapid rate at which humans are changing ecosystems (Vitousek et al., 1997) makes it imperative that genetic diversity be described for as many species as possible so that it can be effectively conserved. Management of extant genetic resources increases the likelihood that populations will be able to successfully respond to their changing environments and thus avoid species extinction (Western, 2001).

Southern Appalachian hardwood ecosystems have been affected by habitat loss and fragmentation (Haskill, 2000), a variety of exotic invasive species (Brown and Peet, 2003), changes in disturbance regimes (Van Lear and Harlow, 2000), and pollution (Woodman, 1987). All of these changes have potentially changed selective pressures acting on populations and drive microevolution within populations. One such ecosystem change is the decline of American chestnut (*Castanea dentata* (Marsh.) Borkh.).This species exerted a significant influence on the functioning of eastern North American ecosystems through its impact on nutrient cycling, decomposition, and ecosystem productivity. These processes have undoubtedly been altered following the decline of the American chestnut as a result of chestnut blight (*Cryphonectria parasitica* (Murill) Barr) (Ellison et al., 2005).

Northern red oak (*Quercus rubra* L.) (NRO) was an important associated tree species in American chestnut dominated forests (Keever, 1953) and in many post-blight southern Appalachian forests, northern red oak has begun to occupy a dominant overstory niche, similar to that once filled by the American chestnut (Kubisiak and Roberds, 2003,

Oak et al., 1991). NRO is an early- to mid-successional, moderately shade tolerant species (Abrams, 1992; Lorimer, 1984) whose regeneration requires a gap or edge environment located on mesic sites, with sparse understory vegetation. These conditions were historically created through gap dynamics produced by fallen American chestnut, fire and possibly damage caused by enormous passenger pigeons flocks (Ellsworth and McComb, 2003) that cleared the forest understory, suppressed common competitors (Crow, 1988) and contributed to creating gaps needed for successful NRO regeneration. The diversity and magnitude of these micro-environmental changes over the past century have potentially altered selection pressures acting on NRO populations, changed the genetic diversity of NRO populations, and possibly lowered the relative fitness of NRO regeneration.

Northern red oak has a broad geographic range (Burns and Honkala, 1990), has significant economic importance (Luppold and Thomas, 1989), and is considered a keystone species due to its dominance in the overstory and volume of hard mast that it produces (Wolff, 1996). However, current NRO regeneration is not successfully reaching the overstory at levels needed to sustain its niche in the ecosystem (Buckley et al. 1998). Harvesting of NRO in the Southern Appalachian region is widespread and important to the regional economy. Some studies have shown that different harvest methods can alter the genetic diversity of hardwood tree species (Buchert et al., 1997; Lee, et al. 2002). Because the adaptive potential of a species can depend on within-species genetic diversity (Reed and Frankham, 2003), it is important to know how micro-environmental changes associated with harvesting could be impacting the genetic composition and structure of NRO populations. Due to the ecological and economic importance of NRO, it

is important to understand the genetic variation present in regional populations, and to determine if the genetic diversity has changed over the past century. Yet, despite the multifaceted importance of NRO and its relatively poor regeneration success, there has been little research concerning the species' genetic diversity as it related to population structure and environmental change over varying time scales (Aldrich et al., 2002; Romero-Severson et al., 2003).

The purpose of this study was to compare NRO genetic diversity between generations that span two major micro-environmental changes. The following two hypotheses were tested:

1) Genetic diversity of NRO populations established before the introduction of the chestnut blight does not differ from that of NRO trees established after the introduction of the chestnut blight.
2) Genetic diversity of NRO populations established in a stand prior to harvest does not differ from the genetic diversity of NRO trees regenerated in the same stand after a harvest.

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CHAPTER TWO: LITERATURE REVIEW

Until recently, the importance of understanding genetic variation of a species has been largely overlooked (Conner and Hartl, 2004). Yet, effective forest management requires knowledge of genetic diversity because different species have different natural levels of genetic variation, and populations require different amounts of genetic variation to remain healthy, functional, viable components of ecosystems (Hamrick et al., 2006). Long-lived woody plant species generally have more genetic variation within species as well as within populations, but have less variation among populations than species with other life forms such as herbaceous plants (Hamrick et al., 1992). Trees have approximately 68% more polymorphic allozyme loci and 45% greater expected heterozygosity (H_e) than herbaceous species (Hamrick, 2004). Woody species with large geographic ranges, outcrossing breeding systems, and wind or animal ingested seed dispersal generally exhibit more genetic diversity within species and populations, but less genetic variation among populations than woody species possessing other trait assemblages (Hamrick et al., 1992). Percent of polymorphic loci, number of alleles per locus and heterozygosity levels are also consistently greater in woody perennial species as opposed to annuals or herbaceous perennials (Hamrick, 2004). Some tree species have shown a positive correlation between heterozygosity and fitness, making maintenance of genetic diversity particularly important, especially given the number of environmental changes that can occur during the course of relatively long lives of trees (Bush and Smouse, 1992).

Genetic Responses to Changing Micro-Environments

When a population inhabits a variable environment and the optimal phenotype of a trait differs relative to changes in that environment, different genetic responses are possible and management decisions may differ depending on the type of response. As local environments change, individuals can either become specialized to different local environments, or they converge on a single fixed phenotype that has a high mean fitness over the environmental gradient. Alternatively, individuals can respond in a plastic way where one genotype can express different phenotypes, and the optimal phenotype for the particular environmental conditions is the one that is expressed (Scheiner, 1998). The evolution of specialized individuals can occur very quickly (years to decades) (Kinnison et al., 2007) compared to the longer time scales (millennia) associated with evolution of species (Darwin 1859). This rapid or micro-evolution can occur when extant populations respond to changing resources, a new biophysical environment, or a new predator or competitor (Reznick and Ghalambor, 2001). Micro-evolution thus facilitates persistence of a species or population in a changing environment, and can result in varying morphology, physiology, and life history characteristics among populations or even among generations within populations (Reznick and Ghalambor, 2001).

In plants, there is widespread evidence of rapid genetic change in response to changing environmental conditions (Hans et al., 1999; Bone and Farres, 2001) such as increased levels of heavy metals (Nordal et al., 1999), disturbance (Hans et al., 1999), herbicides (Heap, 1997), ozone pollution (Davidson and Reiling, 1995), atmospheric CO_2 increases (Ward et al., 2000) and timber harvesting (Law and Salick, 2005). In addition,

invasion by exotic species, fragmentation, and climate change are common causes of habitat change (Schaberg et al., 2008) that disrupt breeding systems causing genetic bottlenecks and increased inbreeding, resulting in population divergence and reduced genetic diversity in a variety of species including wind-pollinated trees (Jump and Penuelas, 2007a and b; Stanton et al., 2009). As genetic diversity decreases the ability of a population to respond to continuing change can decline, and inbreeding depression associated with population size reductions can further reduce the fitness of individuals leading to less viable, more extinction-prone populations (Jump and Penuelas, 2007b; Lande and Shannon, 1996). Therefore, the more genetic diversity a species or population retains, the greater the options available to adaptively respond to changes in the environment. Retention of the potential to evolve makes it imperative that the genetic diversity of a broad range of species is assessed and monitored so that genetic conservation goals of preserving genetic variation, adaptive potential and genetic functioning can be attained.

Micro-evolution at the population level can occur when an exotic invader affects some members of a population more than others, subsequently causing a change in allele frequencies in the native population (Schwartz et al., 2005; Schagberg et al., 2008; Potter et al., 2008; Akimoto et al., 1999). Exotic species that cause the loss of a dominant native species also change selective pressures on the remaining native species through changes in the form, distribution, and abundance of resources that subsequently affect competition, create new niches, and modify the ecosystem for the remaining species (Carroll, 2007). Although there is little literature regarding specific allelic changes resulting from the loss of a dominant species due to exotic pests, the extensive ecosystem

changes are well documented (Ellison et al., 2005). For example, the decline of whitebark pine (*Pinus albicaulis* Engelm.) as a result of the introduced pathogen *Cronartum ribocola* (J.C.Fisch.) has altered watershed hydrology, wildlife population dynamics, and succession (Mattson et al., 2001), and the decline of eastern hemlock (*Tsuga canadensis* (L.) Carriere) due to the hemlock woolly adelgid (*Adelges tsugae* Annand.) will likely lower diversity of benthic macroinvertebrates in streams where the riparian area is dominated by hemlock (Snyder et al., 2002). The loss of the American chestnut (*Castanea dentata* (Marsh.) Borkh.) due to the introduced pathogen *Cryphonectria parasitica* (Murill)Barr) is known to have altered plant, animal and microbial species assemblages, forest gap dynamics, and forest nutrient cycling (Ellison et al., 2005). For example, American chestnut trees were typically over 32 m tall with a crown spread of 31 m (Hagenstein, 1997). When they fell, these trees created large-sized gaps of 740.23 m^2 needed for successful regeneration of species such as northern red oak (*Quercus rubra* L.) (Runkle and Yetter 1987). Because no tree species currently extant in southern Appalachian hardwood forests reaches the size once attained by the American chestnut, the average size of tree fall gaps has declined over the past century, potentially changing selection pressures associated with the amount of light available for regeneration. Individuals that fail to adapt to these relatively rapid micro-environmental changes will be lost and the subsequent population bottleneck will likely result in a genetic bottleneck, and potential loss of more species (Falk and Holsinger, 1991).

Fragmentation can change genetic diversity of populations by decreasing or preventing gene flow between members of a once continuous population. As metapopulations become isolated their effective population sizes decline, leaving them

more susceptible to the influence of genetic drift and inbreeding depression (Hall et al., 1996). The degree to which a habitat can be fragmented before it has an effect on the gene flow of the population varies among species. However, even in species with large total populations, fragmentation can cause locally adapted populations to go extinct, possibly taking with them unique genetic resources. Akimoto et al. (1999) reported a reduction in many diversity measures such as proportion of polymorphic loci, number of alleles per locus, total gene diversity, and intra-population gene diversity as a result of fragmentation in wild rice populations (*Oryza rufpogon* Griff.). Population differentiation measures increased over the study period suggesting a reduction in mating and genetic transfer between subpopulations. A study of European beech (*Fagus sylvatica* L.) conducted by Jump and Penuelas (2007b) found reduced allelic richness, increased genetic bottlenecks and decreased numbers of rare alleles in fragmented relative to unfragmented populations in the same region.

Fragmentation can be caused by timber harvesting through changes in micro-environments in the harvested areas. However, literature relating the effect of harvest on the genetic diversity of tree populations shows no single, overarching pattern. Some studies indicate no significant genetic diversity changes following a harvest (Neale, 1985) while other studies reported declines in the genetic diversity of the regenerating populations. These effects vary by species and appear to be related to variables such as breeding system (Ng et al., 2009), density of individuals in stand (Thiago et al., 2008), type of harvest (Neale, 1985), and loss of genetic resources associated with artificial selection of harvested individuals (Schagberg et al., 2008). For example, mahogany (*Swieetenia macrophylla* King) naturally occurs at low densities of ≤ 1 adult individual

per hectare (Wright, 2002) so removal of any individuals during harvesting significantly reduces the number of potential mates available to remnant individuals, thereby reducing the effective population size and increasing the likelihood of losing alleles to random genetic drift (Thiago et al., 2008). Ng et al. (2009) reported genetic diversity declines following selective harvesting in the outcrossing *Shorea leprosula* (Miq) whereas genetic diversity did not change in the apomicticly reproducing autotetraploid *Shorea ovalis* (Korth.) ssp. *sericea* (Dyer). Selectively harvested *Quercus tiaoloshanica* (Chun & W.C.Ko) populations have experienced a 1% decline in effective number of alleles per locus and clearcut stands experienced a 2% decrease in the number of effective alleles per locus compared to unharvested populations (Zheng et al., 2005). Finally, when eastern white pines (*Pinus strobus* L.) were selectively harvested, poor natural regeneration rates resulted in a loss of alleles (Rajora et al., 2000).

In addition to micro-environmental changes associated with timber harvesting, humans have modified disturbance regimes related to fire thus changing selective pressures associated with interspecific competition. Disturbance has been cited as an important factor in the stability of oak-hickory ecosystems (Taylor and Lorimer, 2003; Loftis, 1990; Lorimer et al., 1994) and one theory for the cause of oak decline in Appalachian hardwood forests is the change in frequency and timing of forest fires (Abrams, 1992). For the majority of the past 4000 years, Native Americans shaped the ecology and species composition of the Southern Appalachians through their use of selectively set fires (Delcourt and Delcourt, 1997) at frequencies of 1.7 to 11.1 years (McEwan et al., 2007). In ecosystems with frequent fire regimes, many species have genetic adaptations such as thick bark to protect the vascular cambium from heat damage

and seeds requiring heat to germinate (Bond and Van Wilgen, 1996). These adaptations can potentially disappear if they are not needed due to fire suppression. Ripple and Larson (2001) observed that piles of coarse woody debris produced by forest fires provide "safe zones" for sexually reproduced aspen seedlings that otherwise would be browsed. This facilitates sexual reproduction and maintains genetic diversity in the typically asexually reproducing aspen. It has also been demonstrated that many perennial herbs produce asexually during fire free intervals, but switch to a sexual mode of reproduction following fire events, allowing for the recruitment of new genetic individuals (Romme et al., 1995). Finally, studies on prescribed burning in oak forests have been shown to increase the size and amount of oak regeneration which prevents declines in fitness associated with inbreeding (Brose et al., 2001).

Changes in fire regimes associated with Euro-American colonization of North American are due to fire suppression (Moritz et al., 2009; Abrams 1992), resulting in buildup of fuel that supports larger, hotter fires than those that historically burned, landscape patchiness has been reduced and species composition, structure, and age distributions have been altered (Baker, 1993; Parsons and DeBenedetti, 1979). Species that are less shade tolerant but more fire tolerant such as *Quercus* spp. (Taylor and Lorimer, 2003) but lack the ability to genetically respond to changing conditions associated with fire suppression will be lost (Pierce et al., 2008; Tybirk and Strandberg, 1999; Peterken, 1996; Parsons and DeBenedetti, 1979).

Climate change has also modified fire-related disturbance regimes, and climate change is expected to alter growing seasons, change patterns of resource allocation and, therefore, drive changes in ecosystem functioning. The alteration of such fundamental

ecological components may exert a selective pressure on adaptive traits selected for over thousands of years, or over a single generation. The conservation of as much genetic variation in a species as possible is important because of the unknown nature of climate change, how species ranges will shift, or how much genetic variation is needed to allow a given species to successfully adapt to these changes. Gathering as much information as possible regarding the genetic structure, composition and function of populations may aid in the conservation of genetic diversity that allows species to successfully adapt to changing ecosystems and species ranges. Informed decisions would then be able to be made with regard to things like the necessity and magnitude of manual transfer of genetic material (pollen transfer, seed/seedling planting, etc.) (McLachlan, et al., 2007). Without consideration of the genetic component of species population structure, the task of maintaining healthy viable populations would be in some cases a “shot in the dark” (Ledig 1993).

It is important to keep in mind that environmental changes do not always result in genotypic differentiation (adaptation) or extinction due to lack of adaptation (DeJong, 2005). Some species can respond via phenotypic plasticity where a variety of phenotypes can be expressed from a single genotype in response to different environmental conditions. Plasticity is an important means of tolerating spatial and temporal environmental variation, especially for sessile organisms such as plants (Schlichting and Levin, 1986; Bradshaw, 1965). Phenotypic plasticity is common in many taxa (Schmalhausen, 1949) and can allow a single genotype to survive while maintaining a high level of fitness, despite environmental change (Sultan, 1995). Traits that are phenotypically plastic can often tolerate environmental change outside the range

normally encountered by the population. For example, exotic species that are plastic are more successful invaders across a wide range of environmental conditions (Hulme, 2008). The degree of phenotypic plasticity of traits, as well as number of plastic traits varies between species (Kramer, 1995; Schlichting and Levin, 1984), between populations of the same species (Schlichting and Levin 1990), as well as within populations (Kramer, 1995). Schlichting and Levin (1990) concluded overall differences in plastic responses between populations were most likely the result of small differences in the plastic response of a number of traits as opposed to large changes in a few traits.

Phenotypic plasticity is under selection pressure (Wells and Pigliucci, 2000), particularly in species where the environment strongly influences genotypic expression of a trait, and when inter-generational changes in the environment are large and predictable (Scheiner, 1998). However, the selective response is complex. Rarely do traits converge on genetic specialization or on high levels of phenotypic plasticity; instead, the varied selective responses of different loci lead to mixed genetic outcomes. A very high level of phenotypic plasticity has the highest *global* fitness, because individuals match the optimum phenotype throughout their species range. However, this high level of phenotypic plasticity is usually not attained because of the adaptation of species to *local* conditions in a spatially-structured environment. Therefore, in populations with high migration rates, phenotypic plasticity is highly favored (Scheiner, 1998).

Ecological Genetics of Northern Red Oak

Northern red oak (*Quercus rubra L.*) is an ecologically and economically important hardwood tree species native to the eastern half of North America (Burns and Honkala, 1990). Many forests in western North Carolina have been classified as an oak-

hickory *(Quercus-Carya)* climax community. This community was prevalent across the landscape prior to extensive European colonization and exploitation (McDonald et al., 2002). Pollen data collected from Horse Cove Bog in Highlands, North Carolina indicate that since the bog began accumulating peat approximately 3550 years ago, the compositional abundance of oak pollen has remained constant at around 35% of total pollen collected. This suggests that oaks have been a dominant and stable component of Southern Appalachian upland ecosystems since this time (Delcourt and Delcourt, 1997). Pollen data also indicate that prior to the last ice age northern red oak (NRO) had a range of a comparable size to its current one. As the Wisconsin ice sheets advanced, the range of NRO was reduced in size and extended further south in the US, followed by recolonization northward as the ice sheet retreated. As a result of a genetic bottleneck, northern populations are "younger" and less genetically diverse than the southern populations as (Schlarbaum et al., 1982).

Despite their long history in the Southern Appalachians, oaks are currently experiencing a decline in regeneration, (Soucy et al., 2004; Lorimer, 1993; Loftis, 1990; Lorimer et al., 1994). For example, in test plots located in the Duke University forest in North Carolina, major oak species show a 50% decline in regeneration over the 70 years compositional data has been collected (McDonald et al., 2002). Increasingly, stands are comprised of older individuals in the overstory with few seedlings or saplings in the understory (Aldrich et al., 2005b). If this trend continues, the reduction in population sizes of NRO will likely lead to declines in genetic diversity resulting in increased inbreeding and subsequent loss of fitness and loss of the species as a dominant overstory component in the forest. Regeneration that has lower fitness will not be able to compete

with the increasing abundance of red maple (*Acer rubrum* L.) (McDonald et al., 2002) and other hardwood species that are more shade tolerant than oak (McClune and Cottam, 1985).

Northern red oak is adapted to early to mid-successional conditions where there are moderate levels of shade (Abrams, 1992; Burns and Holanka, 1990; Lorimer, 1985), and its regeneration is adapted to gaps or edge environment located on mesic sites, with sparse understory vegetation. The species is adapted to frequent fire and other disturbance agents that clear the forest understory, increase light availability, and suppress competitors of the NRO regeneration (Green et al., 2010; Ellsworth and McComb, 2003; Huddle and Pallardy, 1999; Lorimer et al. 1994; Crow, 1988; McClune and Cottham, 1985). Mature NRO has thick bark capable of protecting it from fire damage (Hengst and Dawson, 1994), and seedlings initially favor below ground carbon allocation (Lorimer, 1985) allowing them to re-sprout successfully after shoot dieback (Brose and VanLear, 1998). If some NRO seedlings are genetically predisposed to be more shade tolerant than others and able to survive more effectively in closed canopy forests, natural selection in fire suppressed ecosystems may favor these more shade tolerant NRO individuals and their associated alleles.

NRO has been documented to exhibit differences in adaptive traits throughout its geographical range. Traits that vary geographically include growth rate, drought resistance, cold tolerance, time of flushing and leaf senescence (Kriebel, 1993). In addition to geographic variation, there is evidence of population differentiation most likely due to adaptation throughout the geographic range (Sork et al., 1993b). For example, a study by Magni et al. (2005) reported strong genetic structure among

populations with G_{ST} values averaging 0.46. G_{ST} approximates F_{ST} and Conner and Hartl (2004) consider F_{ST} values greater than 0.25 as indicating very high differentiation. Magni et al. (2005) detected a strong latitudinal gradient in NRO population differentiation, northern populations (the younger ones, inhabiting areas most recently glaciated) were more differentiated than those in the central part of the species range. Populations on a east west gradient did not show great differentiation. In another study at the macro-geographic scale, Sork et al. (1993a) reported high intra-population genetic variation and a moderate level of differentiation among populations (F_{st}= 0.092) for a quantitative trait (insect resistance) that may be under different selective pressure in different microhabitats. They concluded population genetic structure was the result of the large range of NRO combined with the wide variety of environmental conditions and, therefore, selective pressures at the local scale. In contrast, Sork et al. (1993b) assessed fine scale genetic structure of NRO in a single 4 ha site divided into 4 “microhabitats” differing in slope aspect. All populations exhibited high heterozygosity and allelic diversity (F_{IS} = 0.067), but genetic diversity did not vary among aspects (F_{ST}=0.0011). The authors concluded effective population sizes were sufficiently large with adequate gene flow to maintain healthy, genetically viable populations.

The lack of population structuring at small geographic scales could be due to extensive migration. NRO has been predicted to have a large pollen dispersal distance because of its relatively small pollen grains (Docousso et al., 1993), which would contribute to its low among-population differentiation. In northern deciduous forests anemophily (wind pollination) is the dominant means of tree pollination, a trend that can be attributed to several environmental factors such as latitude, species diversity,

vegetation structure, and insular environment (Whitehead, 1969). However, Gleaves (1973) and Knapp et al. (2001) reported concentration of pollen grains in the air sharply declined with increasing distance from a source and that despite the copious amount of pollen produced by wind pollinating trees and the presence of pollen grains found a considerable distance from the source, populations can often be pollen limited. Therefore, population structuring can occur due to lack of migration even between adjacent populations.

Measuring Genetic Diversity

There are a variety of molecular markers that can effectively evaluate genetic diversity at various hierarchical levels, including within and between both individuals and populations. The question of which type of molecular marker is most appropriate in a given situation should be addressed by asking: (1) How much genetic polymorphism is required to detect genetic structure? (2) What analytical and statistical approaches are available? (3) How much time and economic resources are required for each technique? (Parker et al., 1998). Effective markers of ecological genetic analyses provide genetic data that allow the total number of alleles to be scored across all populations at a given locus, calculation of observed and expected heterozygosities, calculation of deviations from Hardy–Weinberg expectations, Wright's F-statistics (Weir and Cockerham, 1984), and number of alleles scored per population. Some of the most widely used molecular marker systems to generate these data include allozymes, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), microsatellites (or SSRs), isozymes, and amplified fragment length polymorphisms (AFLPs).

Allozymes are nuclear encoded enzymes and are often polymorphic, making them suitable for the quantification of population genetic variation. However, because allozymes reflect a variation in protein coding, they may be selectively constrained in ways other neutral marker systems are not (Aagaard et al., 1998). This selection pressure acting on allozymes has the potential to cause balancing selection resulting in an underestimate of allelic difference (Altukhov, 1991) because some species will be monomorphic at most allozyme loci. Furthermore, even species that are generally highly polymorphic at allozyme loci often lack sufficient variation to answer questions concerning very fine scale genetic structure. However, allozyme data are comparatively inexpensive and simple to obtain once allozymes for a particular species have been identified (Parker et al., 1998).

RFLP's were the first DNA markers to be developed and used by population biologists in the early 1980's (Botstein et al., 1980). RFLPs are highly reproducible and easy to score, but require expensive and labor intensive processes such as radioactive labeling and hybridization (Nagaraju, 2001). RFLPs are most effective at answering population genetic structure questions when populations contain high amounts of genetic variation (Parker et al., 1998).

RAPDs are another tool that can be used to study the genetic structure of a population (Williams et al., 1990). RAPDs are more technically simple than RFLPs, are a more random sample of DNA than allozymes, and can detect finer scale differences than allozymes (Black, 1993; Aagaard et al. 1998). Variation in RAPD's occur because of variance in presence/absence of complementary primer annealing sites in a given genomic region. Visualization of a heterozygote is expressed only as a difference in the

intensity of a band on an agarose gel, as opposed to two different bands. This results in uncertainty of the parental origin of alleles and, therefore, RAPDs are not particularly useful for population genetic studies (Parker et al., 1998). Other problems with RAPD technology include random co-migration of bands at different loci which can result in inaccurate data, and the necessity for a very exacting protocol to ensure reproducibility (Black, 1993; Nagaraju et al. 2001).

AFLPs are based on selective PCR amplification and electrophoresis of restriction fragments from a total digest of primarily genomic DNA (Vos et al., 1995; Meudt and Clark, 2007). They have been used successfully in population genetics as well as in population assignment (Meudt and Clark, 2007). AFLPs are dominant markers that have many genome-wide di-allelic loci. Each individual locus has little discerning power, but when many AFLP loci are considered together, they gain statistical power (Meudt and Clark, 2007). Studies comparing AFLPs to microsatellites (Jump and Penuelas, 2007b; Woodhead et al., 2005) found comparable population pairwise F_{ST} values, but differentiation was consistently higher for AFLPs. A hierarchal analysis of molecular variance found that AFLPs also distinguished greater partitioning of variation between populations, while the microsatellites detected more within population variation

Microsatellites (or simple sequence repeats, SSRs) have been recognized in eukaryotic genomes for over 30 years (Hamada et al., 1982). They are developed by screening genomic libraries with probes composed of highly conserved oligonucleotides. The resulting loci are analyzed by PCR amplification followed by gel electrophoresis (Parker et al., 1998). Initially, they were not thought to be of any particular interest because they are non-coding regions of DNA. Following the advent of PCR which

greatly facilitated microsatellite use, their utility has become more and more apparent, particularly for describing population genetic structure (Jarne and Lagota, 1996; Slatkin, 1995). Microsatellite markers are co-dominant and most commonly composed of dinucleotide, trinucleotide or tetranucleotide tandem repeats, although the unit of repetition can be anywhere from one to five base pairs (Jarne and Lagota, 1996). Pedigree analyses of microsatellite loci have indicated co-dominant, neutral, mendelian inheritance. Microsatellite alleles are distinguished from one another based on their fragment length and alleles can differ from one another by as little as a single insertion or deletion of a base pair. In general, microsatellite loci are highly polymorphic in natural populations, where a single locus could have as many as 30-50 different alleles associated with it. The average expected heterozygosity in natural populations is often well above 50%, and in some instances can peak at close to 100% (Jarne and Lagota, 1996).

Compared to allozymes, microsatellites have a rapid mutation rate. Observed values for the mutation rate of microsatellites are two or three orders of magnitude greater that that known for allozymes (Jarne and Lagota, 1996). The most prevalent theory for the mechanism of microsatellite mutation is that of polymerase slippage at DNA replication. This mechanism increases or decreases the previous number of repeats by one unit and results in fragment length differences of even numbers of nucleotides. Other mutational processes such as unequal crossing over also play a role in microsatellite mutation (Jarne and Lagota, 1996). The high mutation rate found in microsatellites makes them ideal for population genetics studies because closely related individuals and populations can be distinguished from one another. Because of their high variability and rapid mutation, microsatellites are very useful tools for evaluating genetic

structure within and among populations. Genetic structure models based on microsatellites assume that two alleles of the same size are identical by descent (IBD), because they descended without mutation from the same ancestral allele.

Initially it was thought that microsatellite primers were unlikely to amplify in members of taxa closely related to the taxon for which the primers were developed; however more recent studies have shown that this occurs more often than was previously thought (Parker et al., 1998). For example, a study conducted by Aldrich et al. (2003b) found that of 30 microsatellite primers developed for northern red oak, 12 (40%) amplified and were polymorphic in the European white oaks *Quercus petraea* ((Mattuschka)Liebl.) and *Quercus robur* (L.). Four of the microsatellite loci also amplified and were polymorphic in Chinese chestnut (*Castanea mollissima* Blume), despite the relatively great phylogenetic distance between the taxa.

Issues affecting the reliability of microsatellite techniques are false alleles (PCR generated alleles), false homozygotes (Goossens et al., 1998), and erroneous allele length attribution (Baldoni et al., 2009). PCR generated alleles are most prevalent when the amount of template DNA going into the PRC reaction is very small (Goossens et al., 1998), whereas erroneous allele attribution occurs independently from amount of PCR template (Baldoni et al., 2009). False alleles occur when an allelic peak is detected on an electrophorogram but instead of representing the genotype of the individual at that locus, the peak is either the result of contamination or an artifact of the PCR process (Goossens, et al., 1998). False homozygotes or allelic dropout occurs when PCR conditions amplify of only one target molecule (Goossens et al., 1998). Erroneous allele attribution is the result of inaccurate separation and identification of neighboring alleles. It is most

common in plants where a high amount the genome is di-nucleotide repeats (Baldoni et al., 2009). Baldoni et al. (2009) asserts that approximately 83% of the scoring discrepancies between laboratories considering the same raw microsatellite data are due to miss-assigned alleles. Alleles often do not conform to a perfect 2-, 3- or 4- base pair periodicity (Ewen et al., 2000). Therefore, both automated binning, a process in which a computer program assigns peaks to certain alleles based on a set of rigid guidelines and manual peak calling, are quite fallible (Amos et al., 2007).

Deviation from 2-, 3- or 4- periodicity can be caused by a variety of factors. Fragments with a high GC concentration migrate through the gel differently from fragments with lower GC content, causing the resultant allele periodicities to deviate from the underlying repeat unit (Amos et al., 2007). Also, some microsatellites are in fact compound repeats as opposed to true di- tri- or tetra nucleotide repeats (Ewen et al., 2000). Mutations in the flanking regions can create differences in the observed length of an allele, which can often lead to the broadening of the length distribution of the locus. Alleles have also been documented to change in size increments different from their typical repeat unit (Amos et al., 2007). In addition to confounding variables present in the sample itself, length estimates of a given fragment can vary up to 0.7 bases for every 5° shift in temperature of the laboratory housing the sequencer (Davidson and Chiba, 2003). For example, when considering the electropherograms of two individuals at a single di-nucleotide repeating microsatellite locus observed to have an average length of between 200 and 220 base pairs, it may not be uncommon to observe peaks at 211.13 and 210.95, respectively. It is likely that both of these peaks represent the same allelic variant, however, are these peaks to be scored both as 212, 210, or 211 (in spite of the fact that

the microsatellite is made of *di-nucleotide* repeats)? Or is the peak at 211.13 to be scored as 212 and the peak at and the peak at 210.95 to be scored as 210 despite the likelihood of the peaks representing the same allele? The consequences of these questions have important implications to the study of population genetics. The designation of fragments into different length classes although they represent the same allele has the potential to undermine the accuracy of estimates of allele frequency and allelic richness (Amos et al., 2007). This dynamic can have a profound impact on the analysis of population genetic composition and structure, potentially distorting estimates of key parameters such as heterozygosity (Amos et al., 2007). Although intrinsic problems connected with microsatellite use exist, many of these problems are uniform over loci and samples, making their use still reliable and meaningful.

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CHAPTER THREE: METHODS

Site Selection

The hypotheses were tested using genetic material collected from four different sites located in the Nantahala National Forest in Western North Carolina (Figure 3-1). Sites were at mid-elevations between 1,000 and 1,830 meters, and mid-slopes with east/northeast aspects. Sites were chosen based on U.S. Forest Service (USFS) forest type, predominance of NRO in the overstory, and management records indicating the last date at which the stand was harvested All of the sites sampled were forest cover Type 53 which is the most prevalent type containing a significant percentage of NRO in the Nantahala National Forest (Forest Service Records, 2006). Although cover Type 53 is designated by the Society of American Foresters as predominantly white oak (*Quercus alba* L.), NRO is an important associated tree species within this forest cover type (Eyre, 1980). Each site was located using maps provided by the USFS, followed by a visual evaluation and characterization of species composition and diameter at breast height (1.37 meters from the ground) for each tree in four randomly located 10 m diameter cross section plots.

Sample Collection

Each of the four sites selected contained two stands with different histories based on USFS records. Each site contained (1) a stand that had not been harvested since the removal of the American chestnut in the 1920’s, and (2) a stand where most of the trees were harvested using clearcut or shelterwood methods during the last 50 years.

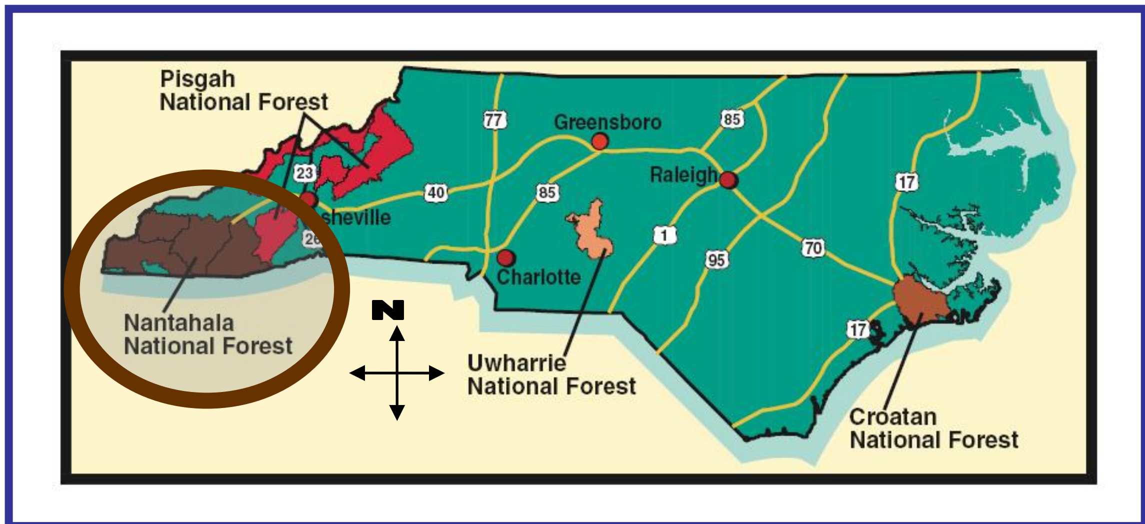

Figure 3-1 Location of Nantahala National Forest in North Carolina

Genetic material collected from stand history (1) was used to test the hypothesis that there are no differences in genetic variation between NRO populations established prior to the removal of the American chestnut compared to NRO regenerated after the removal of the American chestnut. Genetic material collected from stand history (2) was used to test the hypothesis that there is no difference in genetic variation between NRO populations present in a stand prior to harvest compared to those regenerated after harvest.

In each sampled site, 15 to 20 trees growing at least 10 meters from one another were randomly selected to represent each of the four populations of interest: pre- vs. post-harvest in stands that had been recently harvested, and pre- vs. post- blight in stands where only American chestnut was removed in the 1920's. All sampled trees were aged using an increment borer to determine which population they represented. In the harvested stands, only NRO trees that appeared to have regenerated from seed (versus stump sprouts) were included. NRO individuals of stump sprout origin were identified by presence of remnants of a stump at the base of the tree. These trees were excluded from post-harvest populations (personal communication, Dr. Peter Bates, Western Carolina University).

A 3 cm x 3 cm section of bark containing vascular cambium tissue was collected from each tree approximately 0.3 meters from the base of the tree using a hammer and 3 cm chisel. The samples were immediately stored in a cooler on ice for transport to the laboratory where they were stored in plastic bags in a -2º C freezer. Collection methods were based on the recommendations of Dr. Jeanne Romero-Severson (University of Notre Dame), whose laboratory developed the microsatellite markers used in this study.

DNA Extraction

<u>Grinding</u>

A sterile razor blade was used to peel approximately 300 mg of cambial tissue from the bark. The tissue was ground according to DNeasy plant mini-kit protocols (Qiagen, Valencia, CA) modified by increasing the total volume of AP1 lysis buffer used from 400µl to 800µl based on the methods of Dr. Jeanne Romero-Severson (personal communication). Cambial tissue, a steel impactor bead and 400 µl AP1 lysis buffer (Qiagen, Valencia, CA) were placed in a freezer mill (6750 Freezer Mill Spex Sample Prep, Metuchen, NJ) filled with liquid nitrogen and macerated using the following parameters: pre-cooling 20 seconds, grinding 1 minute, cooling between grinding periods 20 seconds, using 2 cycles and an impact frequency rate of 5. The grinding vial was removed from the freezer mill and the temperature was allowed to rise to the point where the end plug could be removed by using the extractor. A metal spatula was used to transfer the ground tissue sample to a labeled 1.5 ml centrifuge tube containing 400 µl lysis buffer. All samples were stored at -20° C until extraction of DNA.

<u>Extraction</u>

DNA was extracted using a modification of the DNeasy plant mini-kit (Qiagen, Valencia, CA) as specified by Dr. Jean Romero Severson (personal communication) by increasing the volume of AP2 buffer from 130 µl to 260 µl. The extraction procedure was carried out under a fume hood because β-mercaptoethanol was used to remove phenolic compounds that undergo substitution reactions with nucleic acids (Wilfinger, 2004). A water bath was heated to 65° C and a sealed bottle of AE buffer was placed in it for later

use. Fifteen microliters of β-mercaptoethanol were added to each sample and incubated for 15 minutes at 65º C, to cause lysis of the plant cells (DNeasy Plant Handbook, 2006). Each sample was vortexed 2-3 times during incubation. Samples were centrifuged at 14,000 rpm for one minute using an Eppendorf micro-centrifuge model 5417R (Eppendorf, Hamburg, Gremany) . With a wide bore pipette tip, the supernatant was transferred to a new 1.5 ml centrifuge tube, 260 µl AP2 buffer was added, and tubes were vortexed and incubated on ice for approximately 10 minutes to precipitate polysaccharides, proteins and detergents (DNeasy Plant Handbook, 2006). The contents of each tube were transferred to the QIAshredder mini spin column (lilac) and placed in 2 ml collection tubes. The columns were centrifuged for four minutes at 14,000 rpm. The QIAshredder spin column removes most of the precipitate and cell debris from the solution, although some may pass through forming a pellet at the bottom of the 2 ml collection tube (DNeasy Plant Handbook, 2006). Without disturbing the debris at the bottom of the collection tubes, the flow-through was divided approximately in half and transferred to two 1.5 ml centrifuge tubes, 750 µl AP3/E buffer was added to each tube, and tubes were placed at -20º C for ~20 minutes. The sample solution was placed in white spin columns attached to a 2 ml collection tube and centrifuged at 8,000 rpm for one minute. The solution at the bottom of the collection tube was discarded, 500 µl AW wash buffer was added to each spin column, centrifuged at 8,000 rpm for one minute, the flow through was discarded, and each spin column was placed in a new 2 ml collection tube. An additional 500 µl AW wash buffer was added to each spin column, tubes were centrifuged at 8,000 rpm for one minute, the flow through was discarded, 500 µl of 95% ethanol was added to the white spin columns, tubes were centrifuged for one minute at

8,000 rpm, and the flow through was discarded. Tubes were then centrifuged at 14,000 rpm for four minutes to dry the remaining ethanol. Drying the membrane is important because residual ethanol may interfere with subsequent reactions (DNeasy Plant Handbook, 2006). Samples were placed in a test tube rack in fume hood for 30 minutes with caps off to ensure all residual ethanol had dried. To disassociate the extracted DNA from the spin column membrane, 55 µl hot AE elution buffer was placed in each tube, allowed to sit for five minutes, centrifuged for one minute at 8,000 rpm. An additional 55 µl hot AE elution buffer was placed in each tube, allowed to sit for five minutes, and then centrifuged for one minute at 8000 rpm. Finally, 1 µl RNase was added to each sample, and tubes were incubated for 30 minutes at 37º C. Samples were then transferred to 5º C for storage.

DNA Quantification and Dilution

The quantity of DNA extracted (ng/ml) was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and nano-drop software (Thermo Fisher Scientific Inc., Waltham, MA). A spreadsheet was constructed using Microsoft Excel (Microsoft Corporation, Redmond, WA) summarizing the DNA concentration of each of the samples. The formula $C_1V_1=C_2V_2$ was used to determine the volume of sample solution and nano pure water needed for each sample to attain 100 µl of working stock solution diluted to a concentration of 7.5 ng/µl. Each sample was placed into a 96-well plate and diluted to 7.5 ng/µl with nano pure water.

Table 3-1. PCR master mix components and concentrations

Master Mix Component	µl per reaction
Sterile Water	5.56
10x Buffer	1.00
$MgCl_2$	0.20
dNTPs 1.25mM each	1.60
Forward Primer (10pmol)	0.30
Reverse Primer (10pmol)	0.30
Taq (HotStar)	0.04
Template DNA	1.00

Polymerase Chain Reaction

Polymerase chain reactions (PCR) were carried out in a 96 well plate using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Concentrations of master mix components are listed in Table 3-1. Primer sequences for 9 (GA)n microsatellite loci developed by Aldrich et al. (2002) were used in this study (Table 3-2). All primers were made by Applied Biosystems (Carlsbad, CA) as custom labeled fluorescent oligo primer pairs. Each forward primer sequence was bound to a fluorescent dye for visualization (6-FAM, NED or HEX). The PCR protocol for each primer was 95° C for 5 minutes, 50 cycles of 94° C for 30 seconds, annealing temperature (Table 3-2) for 45 seconds, 72ºC for 10 minutes, and finally 5° for 5 minutes.

The PCR product was not diluted prior to visualization and was mixed with a solution of Hi-Di Formamide and a ROX 500 (Applied Biosystems, Carlsbad, CA) sizing standard. The size of DNA fragments was determined using a Applied Biosystems 3130 genetic analyzer (Carlsbad, CA) using Applied Biosystems Data Collection software (Carlsbad, CA). Visualization of the microsatellite peaks was accomplished using GeneMapper software (Applied Biosystems, Carlsbad, CA), and scoring was done manually (Amos et al., 2007). Alleles were distinguished from one another based on the number of base pairs present in each fragment.

Data Analyses

The genetic diversity of pre-blight NRO populations in stands that were not been logged since the decline of the American chestnut was compared to the genetic diversity of post-blight populations found in the same stands to determine if genetic diversity

Table 3-2. Primer names, 5'-3' sequences and annealing temperatures

Primer Name	Primer Sequence (5'-3')	Annealing Temperature (T_a)
quru-GA-0E09	*TGCCATCCCTATACACAACCA CCTCCATCACAAAGTTGCC	53ºC
quru-GA-0M07	*TTTAGCATCACATTTCCGTT TTTTGTGTCATCCGGTATTA	45ºC
quru-GA-1C06	*CAAATAAATATTGTGGGGTTCA GGAGGGGATCCGGAAAA	50ºC
quru-GA-1F02	*CCAATCCACCCTTCCAAGTTCC TGGTTGTTTTGCTTTATTCAGCC	50ºC
quru-GA-1F07	*CCGGTCAAAGAAGTTATCAGA GGGTGGATTGGGTTTCTACCTA	58ºC
quru-GA-0C11	*ATACCCAGCTCCCATGACCA TCCCCAAATTCAGGTAGTGT	53ºC
quru-GA-2F05	*CCGCTTCGTGACGATTATTC GAGGTTTGGAGGAGAGATCATTCT	53ºC
quru-GA-2M04	*GGAGAGGACGGGATGCC TACTATGTCAGCCGGATG	56ºC
quru-GA-0C19	*TTAGCTTTTACGCAGTGTCG CGGCTTCGGTTTCGTC	50ºC

differed between the two groups. The genetic diversity of populations present in harvested stands prior to the harvest was compared to the genetic diversity of populations regenerated in the same stands to determine if genetic diversity differed between these two groups. The genetic diversity of harvested stands vs. that of unharvested stands were compared to one another to determine if there was a difference in the genetic diversity of stands that had undergone harvest in addition to the loss of the American chestnut to those that did not undergo additional harvests after the loss of the chestnut. Populations were compared using Genepop (Raymond and Rousset, 1995a; Rousset, 2008), Popgene (Yeh and Boyle, 1997), Arlequin (Excoffier et al., 2005) and Microsatellite Toolkit software (Park, 2001). ANOVAs were used to compare within-stand pre- and post-populations for each hypothesis (Conner and Hartl, 2004; Weir and Cockerham, 1984). The Microsatellite Toolkit software was used to organize data and convert it into formats readable by Arlequin and Genepop, and it was also used to calculate genetic statistics including observed heterozygosity (H_o), expected heterozygosity (H_e), mean number of alleles per locus (A), and base pair size range.

Genepop 4.0.10 was used to confirm calculations of H_o, H_e and A, and to calculate Hardy-Weinberg equilibrium and F-statistics. (Cockerham, 1973; Weir and Cockerham, 1984; Slatkin, 1995). F_{is} was calculated as $(Q_1-Q_2)/(1-Q_2)$, where the Qs were probability of identity in state of pairs of genes either within (Q_1) or between (Q_2) individuals within subpopulations.. F_{st} was calculated as $(Q_2-Q_3)/(1-Q_3)$, where Q_3 is the probability of identity in state of pairs of genes between subpopulations. Hardy-Weinberg exact tests were based on an algorithm described in Guo and Thompson (1992). Genepop was also used to calculate genic differentiation (Raymond and Rousset, 1995b), and

genotypic differentiation (Raymond and Rousset, 1995b). Genic differentiation was computed using the exact G test and compares the distribution of alleles in populations. The null hypothesis for this test was '*alleles are drawn from the same distribution in all populations'.* Genotypic differentiation was also computed using the exact G test and compares the distribution of diploid genotypes in populations. The null hypothesis used by this test is '*genotypes are drawn from the same distribution in all populations'.*

PopGene was used to calculate total number of alleles and effective number of alleles (Ae= 1/[1-He]).The number of private or unique alleles was calculated by hand using data tables generated by Microsatellite Toolkit. Private alleles are alleles that are only found in one study population. In general, the greater the gene flow between populations, the fewer the private alleles (Allendorf and Luikart, 2007). Latent genetic potential (LGP) (Bergmann et al., 1990) was calculated based on the difference between total number of alleles and effective number of alleles summed over all loci. This measure considers allelic richness as a function of the relative number of rare alleles, as rare alleles may contain much of the genetic potential to adapt to ecological change (Mossler et al., 2003).

Arlequin was used to conduct analyses of molecular variance (AMOVA). AMOVA works with molecular markers such as microsatellites to estimate the amount of molecular variance at various levels of user designated population structure such as between groups of populations, among populations within groups, among individuals in a population, and within individuals (Gupta, 2007). This analysis creates a clearer picture of the sources of molecular variance within a population or group of populations.

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CHAPTER FOUR: RESULTS

All nine microsatellite loci were highly polymorphic, ranging from 10 alleles at the 2M04 locus to 55 at the 1F07 locus (Table 4-1). A total of 294 alleles were detected over the nine loci and 251 samples (Table 4-1). Averaged over all sites and primers, the observed and expected heterozygosities were 0.4056 and 0.5523, respectively. Eight of nine primers and the average across all primers significantly deviated from Hardy-Weinberg equilibrium ($p<0.01$), and indicated a heterozygote deficiency (Table 4-1).

Pre- versus Post-Blight Populations

The total number of alleles and number of private alleles did not differ between pre- and post-blight populations ($p= 0.8849$). Mean number of alleles per locus ranged from 8.88 to 15.57 and also did not differ among pre- and post-blight populations ($p = 0.8677$) (Table 4-2), but the mean number of effective alleles in pre-blight population value of 8.002, differed significantly from the post-blight populations of 7.207 ($p= 0.0202$) (Table 4-2).

The H_o differed significantly from H_e ($p<0.0001$) for each pre-blight population and when averaged across sites. The same H_o versus H_e results occurred for post-blight populations for each site and overall average (Table 4-2). In all cases, there was a deficiency of heterozygotes, but the differences between H_o and H_e tended to be greater in pre-blight populations (pre-blight $H_o = 0.6095$ vs $H_e= 0.9013$, post-blight $H_o=0.6353$ vs $H_e =0.7678$). In addition, overall average H_e did not differ between pre- and post-blight populations 0.9013 versus 0.7678, respectively, $p=0.2575$ (Table 4-2).

Table 4-1. Microsatellite DNA primer loci genetic parameters[1] for northern red oak in Western North Carolina.

Microsatellite locus	Repeat Unit	Total no. of alleles	Allele size range (bp)	H_o	H_e	F_{IS}	HW p-values
0E09	$(GA)_{16}$	39	172-220	0.3715	0.6937	0.4651	0.0000
1C06	$(GA)_{29}$	28	226-276	0.1146	0.2938	0.6114	0.0017
1F07	$(GA)_{22}$	55	234-369	0.5929	0.7800	0.2403	0.0563
2M04	$(GA)_{20}$	10	177-220	0.0474	0.0480	0.0129	0.0000
0C19	$(GA)_{18}$	27	208-246	0.5059	0.6772	0.2534	0.0000
2F05	$(GA)_{21}$	42	229-395	0.3478	0.5844	0.4055	0.0000
1F02	$(GA)_{15}$	42	120-195	0.6245	0.6735	0.0741	0.0000
0M07	$(GA)_{19}$	21	183-289	0.2945	0.4125	0.2919	0.0000
0C11	$(GA)_{15}$	30	191-235	0.7510	0.8072	0.0697	0.0112
Mean	-	**32.6**	-	**0.4056**	**0.5523**	**0.2694**	**0.0079**

[1]bp=base pair, H_o= observed heterozygosity, H_e= expected heterozygosity, F_{IS}= inbreeding coefficient (Wright, 1969; Weir and Cockerham, 1984), HW p-value= Hardy-Weinberg p-values.

Table 4-2. Allelic genetic diversity of pre and post blight northern red oak in four stands in western North Carolina

Population	**Alleles**				**Heterozygosity**				
	A_T	**A**	**A_e**	**Private** (#)	**H_o**	**H_e**	**LPG**	**F_{IS}**	**HW p-value**
Pre-Blight									
Wildcat	106	11.78	8.229	23	0.6513	0.8929	31.9349	0.2655	0.0000
Prentiss	89	12.71	9.425	25	0.6344	0.9199	23.0253	0.3181	0.0000
Dirty John	46	9.20	7.076	5	0.5154	0.9019	10.6155	0.4625	0.0000
Rocky Bald	72	10.29	7.359	5	0.6370	0.8903	23.3301	0.2517	0.0000
Mean	**78.25**	**11.00**	**8.002**	**14.5**	**0.6095**	**0.9013**	**22.2265**	**0.3245**	**0.0000**
Post-Blight									
Wildcat	98	10.89	7.133	15	0.7052	0.8802	33.7999	0.2265	0.0000
Prentiss	109	15.57	9.138	32	0.7319	0.8981	45.0327	0.1853	0.0000
Dirty John	56	9.33	6.113	5	0.4078	0.7418	19.3258	0.4562	0.0000
Rocky Bald	69	8.88	6.443	8	0.6962	0.8813	23.8968	0.1797	0.0000
Mean	**83**	**11.17**	**7.207**	**15**	**0.6353**	**0.7678**	**30.5138**	**0.2619**	**0.0000**
p-value	**0.5083**	**0.8677**	**0.0202**	**0.8849**	**0.6110**	**0.2575**	**0.1900**	**0.1034**	

A_T = total number, A= mean number per locus, A_e =mean effective number of alleles per locus, Private = number of private alleles
H_o = mean observed heterozygosity, H_e = mean expected heterozygosity
LPG = latent genetic potential, Bergman et al. 1990
Fis = inbreeding coefficient, Wright 1969, Weir and Cockerham 1984
p-value compares mean pre-blight value to mean post-blight value for each column

F_{IS} values ranged from 0.1797 to 0.4625 (Table 4-2) with inbreeding values in pre-blight populations generally greater than in post-blight populations, however differences between populations were not significant (p=0.1034). Latent genetic potential (LGP) was calculated to be 22.2265 in pre-blight populations and 30.5138 in post-blight populations. Although LGP was greater in post-blight populations, these values were not found to significantly differ (p=0.1900) (Table 4-2).

Genic distribution of alleles varied significantly between pre- and post-blight populations for each site and overall, with p-values ranging from 0.0000 to 0.0461 (Table 4-3). Twelve percent of alleles differed in frequency of at least 10% between pre- and post-chestnut blight generations (data not shown). Despite the differences in allelic composition, genotypic differences as reflected in observed heterozygosity did not differ significantly (p= 0.3514) between pre-and post-blight populations (Table 4-3). F_{ST} values comparing pre-blight and post-blight populations were very low and approached zero for each site (Table 4-3).

Results of AMOVA showing the partitioning of genetic variance is summarized in Table 4-4. The majority of the genetic variation was tree-to-tree within sites ($p < 0.01$), and genetic differences among sites and between pre- and post-blight populations accounted for only a small amount of the distribution of genetic variance. Although most of the genetic variation was within individuals (97.59%) and within each site (16.32%), pre- versus post- blight populations did account for ~ 5% percent of the total amount of genetic variation present.

Table 4-3. Genetic differences in pre- versus post-blight populations of northern red oak in four stands in western North Carolina.

Population	**Genic (p-value)**	**Genotype (p-value)**	**F_{ST}**
Wildcat	0.0025	0.4521	-0.0003
Prentiss	0.0000	0.0691	0.0078
Rocky Bald	0.0099	0.1088	0.0050
Dirty John	0.0461	0.7756	-0.0125
Overall	0.0146	0.3514	0.0000

F_{ST}= Indicator of population structure with values closer to zero indicating less structure, Wright, 1969, Nei, 1977.

Table 4-4. Distribution of genetic variation in pre- versus post-blight northern red oak populations in western North Carolina.

Source of Variation	**df**	**Variance Component**	**Percentage of Variation**	**p-value**
Pre- versus Post-blight	1	0.08483 Va	4.90	0.1681 (±0.0118)
Among Sites	6	-0.32583 Vb	-18.81	1.0000 (± 0.0000)
Tree-to-tree within Site	131	0.28275 Vc	16.32	0.0000 (± 0.0000)
Within Individuals	139	1.69065 Vd	97.59	0.1466 (±0.0103)
Total	277	1.73241		

Significance tests (1023 permutations)

Pre- versus Post-Harvest Populations

Mean number of alleles per locus in sites that had been harvested ranged from 4.67 to 12.86 and did not significantly differ between pre- and post-harvest populations (p = 0.763) (Table 4-5). Mean total number of alleles ranged from 69.25 in pre- harvest populations to 73. 25 in post-harvest populations, however these values did not statistically differ (p= 0.5619). Mean total number of effective alleles ranged from 6.720 in pre-harvest populations to 7.2185 in post- harvest populations, however these values were also not statistically different from one another (p= 0.6613). In addition, the number of private alleles also did not differ between pre- and post- harvest populations (Table 4-5).

Estimates of H_e in all cases exceeded the estimates of H_o, and H_o significantly differed from H_e (p= 0.0001) for each pre- harvest population and averaged across sites. The same H_o versus H_e results occurred for post-harvest populations for each site as well as overall average (Table 4-5). Observed heterozygosity (H_o) ranged from 0.3228 in to 0.7715 but differences among pre- and post-harvest populations did not differ significantly (p = 0.1470, Table 4-5). In all cases there was a deficiency of heterozygotes, however the difference between H_o and H_e tended to be greater in the pre-harvest populations. F_{IS} values ranged across the sites from 0.1412 to 0.5004 with the average in pre-harvest populations (0.2883) greater than in post-harvest populations (0.2690). However no statistical differences was found (p= 0.8637) (Table 4-5). Latent genetic potential (LGP) was calculated to be 21.4895 in pre-harvest populations and 21.8227 in post-harvest populations, and these values were not found to significantly different from one another (p=0.8930) (Table 4-5).

Table 4-5. Allelic genetic diversity of pre- and post- harvest populations of northern red oak in western North Carolina

Population	Alleles				Heterozygosity			F-Statistics	
	A_T	A	A_e	Private (#)	H_o	H_e	LPG	Fis	HW
Pre-Harvest									
Wildcat	98	12.25	9.594	13	0.6426	0.9107	21.2512	0.2837	0.0000
Prentiss	81	11.57	6.695	13	0.6858	0.8660	34.1356	0.2071	0.0000
Dirty John	21	4.67	3.359	2	0.3228	0.6884	4.2005	0.5004	0.0000
Rocky Bald	77	11.00	7.233	11	0.7376	0.8928	26.3706	0.1619	0.0000
Mean	**69.25**	**9.87**	**6.720**	**9.75**	**0.5972**	**0.8411**	**21.4895**	**0.2883**	**0.0000**
Post-Harvest									
Wildcat	86	9.26	7.364	17	0.5171	0.8037	19.7237	0.3244	0.0000
Prentiss	90	12.86	8.653	17	0.7603	0.9045	29.4319	0.1481	0.0000
Dirty John	38	7.50	5.568	3	0.3678	0.7377	10.1641	0.4623	0.0000
Rocky Bald	79	11.29	7.289	6	0.7715	0.9030	27.9712	0.1412	0.0000
Mean	**73.25**	**10.23**	**7.2185**	**10.75**	**0.6042**	**0.8372**	**21.8227**	**0.2690**	**0.0000**
p-value	**0.5619**	**0.763**	**0.6613**	**0.6695**	**0.1470**	**0.9539**	**0.8930**	**0.8637**	

A_T = total number, A mean number per locus, A_e mean effective number of alleles per locus, Private = number of private alleles
H_o = mean observed heterozygosity, H_e = mean expected heterozygosity
LPG = latent genetic potential, Bergman et al. 1990
Fis = inbreeding coefficient, Wright 1969, Weir and Cockerham 1984,
p-value compares mean harvested value to unharvested value for each column

Although there were genic differences between pre- and post-harvest populations at the Wildcat and Dirty John sites ($p = 0.02163$ and 0.02804, respectively), overall there was no significant difference between pre- and post-harvested populations ($p = 0.1437$) (Table 4-6). Genotypic differences between pre- and post-harvest populations also did not vary significantly for any of the four sites or overall (Table 4-6). F_{ST} values for pre- versus post- harvest sites all approached zero

The analysis of the partitioning of genetic variance in harvested areas is summarized in (Table 4-7). The majority of the genetic variation was tree-to-tree ($p < 0.01$), but there was a small percentage of genetic variance associated with pre- versus post-harvest populations (4.46%)

Averaged Pre- and Post-blight populations vs. Averaged Pre- and Post-harvest populations

Trees in the harvested sites represented populations that experienced the cumulative effects of stand changes due to the chestnut blight, plus the effects of harvesting. Analyses to determine if these cumulative effects were resulting in genetic differences revealed that allelic measures of genetic diversity (A, A_e, H_o and H_e) were all slightly higher in the averaged pre- and post-blight populations but, none of these differences were statistically significant (Table 4-8).The same trend was also found for LGP. However, when diversity measures such as genic differentiation, genotypic differentiation and F_{ST} were compared between averaged pre- and post-harvest and averaged pre- and post-blight NRO populations, genetic differences were detected. Genic differentiation had a p-value of 0.0000, genotypic differences had a p-value of 0.02558, and F_{ST} was 0.261 (Table 4-9).

Table 4-6. Genetic differences in pre- versus post-harvest northern red oak populations in four harvested areas in western North Carolina.

Population	**Genic (p-value)**	**Genotype (p-value)**	$\mathbf{F_{ST}}$
Wildcat	0.0216	0.7472	-0.0068
Prentiss	0.1497	0.5062	-0.0056
Rocky Bald	0.3754	0.8768	-0.0065
Dirty John	0.0280	0.5599	-0.0408
Overall	**0.1437**	**0.6725**	**-0.0149**

$\mathbf{F_{ST}}$= Fixation index, Wright, 1969, Nei, 1977.

Table 4-7. Distribution of genetic variation in four harvested northern red oak sites in western North Carolina

Source of Variation	**df**	**Variance Component**	**Percentage of Variation**	**p-value**
Pre- versus Post-harvest	1	0.0787 Va	4.46	0.0498 (±0.0068)
Among Sites	6	-0.3050 Vb	-17.28	1.0000 (± 0.0000)
Tree-to-tree within Site	107	0.1398 Vc	7.92	0.0000 (± 0.0000)
Within Individuals	115	1.8522 Vd	104.9	0.9941 (±0.0033)
Total	229	1.7657		

Significance tests (1023 permutations)

Table 4-8. Allelic genetic diversity of averaged pre- and post-harvest populations and averaged pre- and post-blight populations of northern red oak in western North Carolina

Average Population	A	Ae	Ho	He
Pre- and Post-harvest	15.6	9.13	0.6087	0.8597
Pre- and Post-blight	14.4	8.45	0.5609	0.8696
P-value	**0.3017**	**0.4264**	**0.9511**	**0.8552**

A_T = total number, A mean number per locus, A_e mean effective number of alleles per locus, H_o = mean observed heterozygosity, H_e = mean expected heterozygosity
p-value compares mean harvested value to unharvested value for each column

Table 4-9. P-values of genetic tests comparing averaged pre- and post harvest and averaged pre- and post-blight populations of northern red oak in four harvested areas in western North Carolina.

Genic Differentiation	Genotypic Differentiation	F_{ST}
0.0000	0.02558	0.261

F_{ST}= Fixation index, Wright, 1969, Nei, 1977.

Table 4-10. Tree-to-tree AMOVA comparison between averaged pre- and post-harvest and averaged pre- and post-blight populations of northern red oak in western North Carolina.

AMOVA	Averaged Pre- and Post-blight	Averaged Pre- and Post-harvest
Tree-to-tree within site	16.32	7.92

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CHAPTER FIVE: DISCUSSION

Similar to other studies of woody, wind pollinated tree species with large geographic ranges (Hamrick et al. 1992) and other studies of NRO (Sork et al. 1993; Aldrich et al., 2005), this study found high levels of genetic diversity within individuals and populations (>60% heterozygosity in all stands), but lower levels between populations (Tables 4-4 and 4-7). This pattern in NRO most likely has to do with the life history and modes of dispersal of the species. Species such as NRO that are outcrossing and wind pollinated tend to have the majority of their genetic variation contained within populations, and comparatively little variation between populations (Hamrick et al., 1989).

Allelic Diversity

The lack of difference in allelic richness between pre- and post-blight and pre- and post- harvest NRO populations indicates allelic diversity has been maintained despite harvesting and loss of a foundation species, which likely resulted in micro-habitat changes. American chestnut is known to have exerted a significant influence on ecosystem functioning (Ellison et al., 2005) and in other systems the loss of a foundation species has been reported to drive evolution in associated species (Shuster et al., 2006). The loss of the American chestnut has altered ecological factors such as gap dynamics, light availability, and understory species assemblages in eastern forests (Mikan et al., 1994). The lack of differences in this study imply that ecological impacts of harvesting and the loss of the American chestnut did not change selective pressures enough to

reduce allelic richness in NRO. The pre- and post-harvest results are similar to other reports on the effects of harvest on outcrossing tree species (e.g., Silva et al., 2007; Buchert et al., 1997; Degen et al. 2006). Where the lack of genetic change following disturbance was attributed to regeneration adapted to the micro-environment changes of the disturbed site, the number of reproductive individuals contributing to regeneration was not reduced significantly or, the creation of forest gaps allowed greater long distance pollen flow into the stand thus maintaining the allelic diversity of the stand. Alternatively, NRO could be responding to changes in selective pressure through a plastic response to ecological change (Takahashi et al. 2008). With respect to response to light availability NRO has been found to have an intermediate level of plasticity (Paquette et al., 2007), which could account for the low levels of genetic divergence between populations. It is likely that NRO exhibits traits with a high level of phenotypic plasticity because according to Wells and Pigliucci (2000), phenotypic plasticity can be selected for when the population in question experiences inter-generational changes in the environment that are large. Lowe et al. (2005) also found little genetic impact in species having undergone habitat change, however, when reproductive output and fitness of progeny were considered, significant decreases were detected. This pattern could partially account for the lack of regeneration (McDonald et al., 2002) and reflect lower fitness of NRO in current southern Appalachian forests.

The explanations for lack of genetic differences pre- and post-harvest can also apply to pre- and post- blight populations. However, in contrast to harvested populations effective number of alleles varied among pre-and post-blight populations (Table 4-2). Effective number of alleles is a measure of the allelic evenness (Gregorius, 1978; Kimura

and Crow, 1978) of a population and reaches its maximum when the frequencies of alleles in a population are identical to one another, and approaches a minimum when frequencies of alleles are dissimilar. The distribution of allele frequencies was more uniform in pre-blight populations compared to post-blight populations (data not shown) suggesting that some of the changes in the micro-environment have resulted in changes in selection pressure that are favoring some alleles over others. For example, 12% of alleles differed in frequency of at least 10% between pre- and post-chestnut blight generations. The fact that genic differentiation differed between pre- and post-blight populations suggests that even though heterozygosity levels were similar, the alleles that made up the heterozygosites varied (Table 4-3). Finally, pre- versus post-blight populations did account for ~ 5% percent of the total amount of genetic variation present.

Heterozygosity and F_{IS}

Oaks are outcrossing tree species with low levels of inbreeding and high levels of gene flow (Hamrick and Godt, 1996). F_{IS} values in this study are slightly higher than F_{IS} values found in other studies of wind pollinated trees such as white spruce (*Picea glauca* (Moench) Voss) where Rajora et al. (2005) reported F_{IS}=0.226. Additionally, Jump and Penuleas (2006) reported F_{IS}=0.127 for a European beech (*Fagus sylvatica* L.) population. The deficiency of heterozygotes and high F_{IS} values seen in all populations in this study have been reported in other tree species including black oak (*Quercus velutina* Lamb) (Fernandez-Manjarres et al., 2006), white spruce (Rajora et al., 2005;Tremblay and Simon, 1989; Alden and Loopstra, 1987), Fraser fir (*Abies fraseri* (Pursh) Poir.) (Potter et al., 2008), gaboon (*Aucoumea klaineana* Pierre) (Born et al., 2008), English oak (*Quercus robur* L.) and sessile oak *(Quercus petraea* (Mattuschka) Libel.*)* (Gormory

et al., 2001). The deficit of heterozygotes caused all populations to be out of Hardy-Weinberg equilibrium (Table 4-2), indicating that genetic change is occurring in all the study populations. However, the factors causing change are likely the same in pre- and post-disturbance populations. Possible causes for lower H_o compared to H_e in my study include inbreeding, and Whalund's effect (Rajora et al., 2005). Inbreeding can occur because, statistically it is more likely for a wind pollinated tree to mate with individuals closer to it as opposed to further away. It is also possible that habitat fragmentation has isolated populations from one another and reduced pollen flow into the stands. Finally, the relatively low density of NRO individuals in stands dominated by hickory and white oak, possibly leads to a small effective population or lack of pollen mixing among NRO. The lack of NRO regeneration in the understory (Aldrich et al., 2005) may also have reduced the effective population size in the stands. This dynamic can cause genetic bottlenecks and result in inbreeding in populations. Other studies in oak species (Vakkari et al., 2006) attributed high F_{IS} values and heterozygote deficiencies to Wahlund's effect. Wahlund's effect results from the pooling of samples taken from a large area with significant sub-structuring (spatial or temporal) (Jump and Penuelas, 2007). It is a possibility that the NRO populations in this study were experiencing significant sub-structuring within the stands, possibly due to pollen dispersal patterns.

Interestingly, in all cases, deficiencies in heterozygosity were greater in pre-blight and pre-harvest populations compared to post-blight and post-harvest populations. This may be attributable to increases in wind movement when stand densities were reduced resulting in increased pollen dispersal across the disturbed landscape. Studies have shown that when tree density in a stand is lower, such as in the case of harvest or disturbance,

wind can move more continuously through a stand and increase the dispersal distance of pollen, thereby maintaining the effective population size, and reducing the effect of inbreeding (and heterozygote deficiency) in a population (Robledo-Arnuncio et al., 2004).

F_{ST}

There was no overall detectable structuring between pre- and post-blight populations (F_{ST}=0) or pre- and post-harvest populations (F_{ST}=-0.0149) of NRO as indicated by F_{ST} values. Other studies of NRO have found a similar lack of structuring at this spatial scale. Sork et al. (1993) reported an F_{ST} of 0.011 for adjacent subpopulations, and Schwartzmann and Gerhold (1991) found a G_{ST} of 0.009 for eight populations of NRO in Pennsylvania. In addition, high levels of intra-population homozygosity for highly variable loci, such as microsatellites even when few or no alleles are shared between populations can cause structuring estimates to be low (Hedrick, 2005).

Cumulative Effects of Blight plus Harvest

When all individuals in the blight study populations were combined and compared to all individuals in the harvest study populations, population divergence was detected by the diversity measures of genic differentiation, genotypic differentiation and F_{ST} . In addition to harvest, the harvested stands were also subject to the loss of the American chestnut. It is possible the harvested stands have undergone the genetic changes observed in the pre- versus post-blight comparisons and subsequent harvesting disturbances have lead to population genetic changes not detected at the single generation scale of pre- and post-harvest.

Summary

Though the majority of parameters considered in this study showed little genetic differentiation between pre- and post-chestnut blight populations, effective number of alleles, genic differentiation and AMOVA all showed a small degree of genetic divergence. This infers that it is possible selective pressures in the sampled sites have changed enough over time to precipitate genetic change. The lack of difference could reflect the ability to respond to some of these changes in a plastic way. Little genetic differentiation was also seen between pre- and post-harvest populations. This infers that NRO is already adapted to or is responding in a plastic way to microclimate changes which occur during a harvest, and that harvest alone does not change selective pressures enough to drive evolution in southern Appalachian NRO populations. However, multiple disturbances over time such as loss of American chestnut with harvesting may be resulting in long-term changes.

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