Viability of butternut (Juglans cinerea L) in the Northeastern United States: An assessment of the genetic diversity, health, and hybridization and recruitment of butternut in the Northeast

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VIABILITY OF BUTTERNUT (*Juglans cinerea* L.) IN THE NORTHEASTERN UNITED STATES: AN ASSESSMENT OF THE GENETIC DIVERSITY, HEALTH, HYBRIDIZATION AND RECRUITMENT OF BUTTERNUT IN THE NORTHEAST

BY

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THESIS

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant Biology

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This thesis has been examined and approved.

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For Robert and Heidi, who embody dedication, love and hard work
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ABSTRACT

VIABILITY OF BUTTERNUT (*Juglans cinerea* L.) IN THE NORTHEASTERN UNITED STATES: AN ASSESSMENT OF THE GENETIC DIVERSITY, HEALTH, HYBRIDIZATION AND RECRUITMENT OF BUTTERNUT IN THE NORTHEAST

by

André Boraks

University of New Hampshire, September, 2013

Butternut (*Juglans cinerea*) trees are being extirpated from their natural range by means of an epidemic caused by a fungal pathogen. Widespread mortality is reminiscent of past epidemics on American chestnut (*Castanea dentata*) and American elm (*Ulmus americana*). Understanding the course of contemporary decline of this tree species will provide greater insight on processes of potential extinction and the results of management to prevent it.

This thesis applies an interdisciplinary approach to characterize butternut of the northeastern United States. While there is evidence of weak genetic structuring, butternut appears to have maintained sufficient dispersal to prevent isolation and inbreeding depression. This observed genetic variation is an artifact of past community contiguity. Today, butternuts are recruiting insufficiently to prevent near-term population collapse. Integration of resistant hybrids should be a top priority. Information related in this thesis illuminates voids in our understanding of butternut and should be used to guide conservation policy.
CHAPTER 1:
INTRODUCTION

1.1 Scope of thesis

An understanding of the natural world allows for the anticipation of future events and in turn, aids in better understanding of our relationship within the natural world. Anticipation of change relies on the recognition of patterns that can be used to model natural systems. Traditionally, ecologists focused on patterns created by the tangled web of interactions between species (Falk and Holsinger, 1991). While these webs may be of interest to particular facets of ecology there is little relation between flow webs and community organization (Paine, 1980). The scale of any biological study should be characteristic of the organism under consideration. Populations should be considered within the context of individual interactions rather than a web of who eats who. An individual based approach allows researchers the building blocks to recognize associations within communities. These intraspecific associations are of great importance to applied ecology. For example, the rate and spread of a pathogen depends on the structure and assemblage of a host population (Salathé and Jones, 2010). Understanding the structure and assemblage of a host population relies on an understanding of individual based interaction.

The capacity to typify a community lies in our ability to sample from that community. Careful consideration should be applied to the sampling approach and methods used in any study as these factors can dramatically affect an outcome (Schwartz and McKelvey, 2008). Differing organisms require tailored approaches to study
methodology. The opportunity to study a non-model organism can shed new-light on our understanding of that particular organism, and more generally on natural processes as a whole. Trees live a long time and our ability to study them is hindered by our comparatively shorter life span. Understanding the processes that influence community change in a forest is more difficult when compared to organisms with a shorter lifespan. Trees can reveal community interactions that may not be apparent in organisms with a shorter lifespan, for example, the simultaneous monitoring of individuals and communities. Researchers are provided the valuable option of timely assays that may otherwise be impossible.

The longevity that allows for novel insight is also an obstacle associated with measuring tree communities. Longevity requires a particular approach to understanding processes affecting tree communities. One method of community characterization is to document current patterns of growth and ecology, leading to a better understanding of a particular niche. Predicting how that niche may have historically changed allows for the prediction of how trees may respond to variation in the future, although results from this approach can be limited.

Genetic analysis is an alternative, and somewhat less biased approach to inferring current and historical responses. Similar to a census of age cohorts, genetic patterns can inform researchers of past demographic events. A variety of molecular tools are available for use in genetic analyses. Simple tandem repeats (STR), known as microsatellite, are short repeats of nucleotide base-pairs that vary between individuals. Molecular ecologists use microsatellites to measure the relatedness between and among individuals.
Combined, genetic and ecological analysis leads to informed conclusions. Our ability to characterize communities is fundamental to understanding the natural world around us. This thesis takes an interdisciplinary approach at characterizing a population of butternut trees. Through this work I aim to contribute a new understanding for butternut (*Juglans cinerea*) of the northeast by documenting fine-scale genetics and life history for butternut of Maine, New Hampshire, Vermont, and New York. My interest in this field of work begins at the crossroad of trees and fungi. Members of two vastly different kingdoms, fungi and trees are often found growing in synonymous habitats, fulfilling a variety of relationships with each other. Fungi may exist in parallel with trees as a symbiont, saprophyte, or as part of a disease complex. This thesis analyzes the results of an epidemic caused by a fungus on the butternut tree.

1.2 Study organism

Native to North America, the butternut tree (*Juglans cinerea*) is an economically and ecologically important member of the eastern deciduous forest (Woeste and Pijut, 2009). The natural range of butternut extends from southern New Brunswick and Quebec west to Minnesota, as far south and southwest as Georgia and Missouri (Figure 1.1). One of the most cold-hardy of the *Juglans* species, butternut’s range overlaps with, but extends further north than the black walnut (*J. nigra*) (Rink, 1990). Butternut is not an abundant forest tree, historically contributing 1-3% arboreal pollen (Delcourt, 1979) and is found as sparse stands in association with other mixed hardwoods (Schultz, 2003). Growing to an average height of 30 m tall and 90 cm in diameter, the butternut has a moderate life span of less than 100 years (Farlee et al., 2010).
Butternuts reach maturity around the age of twenty by producing inconspicuous monocious flowers. Primarily a wind pollinated species, male catkins develop asynchronously to female spikes in a process known as heterodichogamy (Gleeson, 1982). Heterodichogamy differs from regular dichogamy in that two mating types are involved (protogyny and protandry) at equal ratio. Both mating types occur simultaneously within a population, the purpose of which is to promote the occurrence of out-crossing. Upon fertilization, oblong nuts develop within the growing season and are shed shortly after leaf-fall (Figure 1.2). The large nuts are dispersed by gravity, scavenging rodents, and water (Rink, 1990), usually within moderate proximity of the seed-bearing mother. Each tree produces a mast bumper crop every two to three years (Ostry and Pijut, 2000).

1.3 Hybridization

The butternut tree, a predominantly outcrossing species, has the ability to naturally hybridize with at least two other exotic congeners. Japanese walnut (J. ailantifolia Carr.) and English walnut (J. regia L.) were introduced to North America during the mid-19th century for use as ornamentals and for nut production (Ostry and Moore, 2007). These exotic Juglans species have naturally hybridized with butternut since their introduction (Ostry and Woeste, 2004), as it is common for woody taxa from Asia to successfully hybridize with its North American congenic (Wen, 1999). The hybridization of disjunct species generally results in extrinsically and intrinsically unfit progeny reducing the likelihood of successive hybrid generations (Mayr, 1992). Whether
or not genetic invasion becomes a concern depends on the fitness and fecundity of successful hybrids.

It has been proposed that hybrids of Japanese walnut and butternut can persist in natural settings (Hoban et al., 2009). Japanese walnuts and related hybrids are generally used as ornamentals or for nut crop production and are usually restricted to fragmented semi-rural landscapes (Ostry and Woeste, 2004). The rate of butternut hybridization is still unknown due to limited range-wide monitoring and difficulties associated with hybrid backcrossing. Rapid genetic invasion is suspected to have not occurred as Japanese walnuts were introduced to North America from Japan around 1870 (Manning, 1978) and pure butternuts are still present in greater abundance (Zhao and Woeste, 2010). Cultivated hybrids of Japanese walnut and butternut (*J. bixby*) are both vigorous and prolific to the extent that concern of genetic invasion has already been raised (Ostry and Woeste, 2004).

1.4 Butternut canker

Currently, the exotic fungus *Ophiognomonia clavigignenti-juglandacearum* (*Oc-j*) Broders and Boland, threatens the butternut tree to the point of extinction. *Oc-j* is the causal organism responsible for butternut canker. Identified in 1976, reports of butternut canker rapidly spread and the fungus is currently found throughout butternut’s entire range. First reports of butternut canker came from Wisconsin, although the possibility of multiple introductions has been raised on account of the speed at which *Oc-j* infected the entire range (Broders et al., 2012). Contributing factors to *Oc-j*’s rapid spread include the movement of infected nursery seed (Andre et al., 2001) and the wind-born nature of
spores (Tisserat and Kuntz, 1983). Nearly all butternuts are infected with butternut canker leading to many instances of population’s reduction by 75% (Ostry et al., 1994). Little, if any genetic resistance exists, furthermore resistance by isolation seems unreasonable due to the apparent long-distance transmission of Oc-j. Resistance to Oc-j is essential for the long-term survival of butternut (Michler et al., 2006).

1.5 Study objectives

The persistence of a species can be anticipated by monitoring the genetic diversity and gene flow among and within the species. Analytical methods can present factors on how a population declines and the types of policies that should be made for management. Monitoring for the viability and persistence of a population is a first approach to managing a species. This thesis approaches the analysis of butternut populations from traditional (census) and modern (genetic) ecological approaches. Results from this research combined with other published studies provide an analysis of butternut covering a variety of locations and sampling resolutions. This study set out to characterize a population of butternut in the northeastern United States by addressing recruitment, health, hybridization and genetic diversity. Furthermore, data presented here aims to compliment studies of a similar nature by applying previously addressed questions to a new location and sampling scheme. Butternut, threatened by an exotic fungus, provides researchers with an opportunity to observe the effects of an epidemic and its relation with population fluctuation and persistence. More practically, this study provides a general health assessment and spatial genetic structuring analysis for application in management.
and conservation by addressing the physical and genetic of northeastern butternut.
Figure 1.1. Native range of butternut (*Juglans cinerera*) modified from Rink et al. (1990). Sampling range indicated by points.
Figure 1.2. The distinctive phenotype of butternut (*Juglans cinerea*) seeds, oblong and densely pubescent. These immature seed display protruding pistils.
CHAPTER 2:
POPULATION GENETICS OF BUTTERNUT IN THE NORTHEASTERN UNITED STATES

2.1 Introduction

*Juglans cinerea*, known as the butternut tree or white walnut, is an economically and ecologically important member of the eastern deciduous forest, native to eastern North America (Figure 1.1). Butternut is a relatively short lived species reaching maturity at 15-20 years and rarely exceeding 75 years in age (Ostry et al., 2003). Relying on wind pollination, butternut trees are heterodichogamous relying on asynchronous development of male and female flowers (Gleeson, 1982). Successful fertilization results in significant seed crops every 2-3 years, the heavy seed requiring dispersal by gravity, water, or scavengers. Long distance dispersal occurs by wind pollination or movement of seed and nursery stock by humans.

A notable disease on butternut was first reported in Wisconsin 1967 (Renlund, 1971). The disease widely known as butternut canker is caused by the ascomycete *Ophiognomonia clavigignenti-juglandacearum* Broders and Boland (*Ocj*). Today *Ocj* threatens the entire range of butternut responsible for mortality rates approaching 80% (Ostry et al., 2003). *Ocj* can infect butternut trees of all ages, often killing saplings more rapidly than mature trees. Initially, butternut canker was not reported in New England and New York (Anderson and LaMadelaine, 1978), however by 1982 *Ocj* was present in the Northeast (Kostichka, 1982). More recent surveys in the Northeast reveal a disease incidence rate of nearly 100% and a mortality rate of 25% (Bergdahl, 2009).

Microsatellite markers are routinely used to help define the genetic structure of
tree populations (Yao et al., 2011). Understanding the drivers of population structuring is important due to its relevance in speciation and extinction events. Defining patterns of genetic structuring for an endangered species is a critical first step to genetic management. Butternut provides a rare opportunity to analyze the effects of in situ pathogenic invasion on forest species population structuring.

The present study uses 6 nuclear DNA microsatellite markers to elucidate fine-scale relationships of butternut in the northeastern United States. The northeastern US approaches the northern distal portion of the butternut's range and includes a vast landscape of favorable growing locations. This study addresses the critical first step of fine-scale genetic structuring as a means to inform policy and genetic management.

Fine-scale genetic studies of butternut's northeastern range are few. Past studies of northeastern populations conclude similar low levels of genetic structuring (Ross-Davis et al., 2008; Morin et al., 2000; Hoban et al., 2010), although a common occurrence of spatial sample clumping may have skewed results. Furthermore, previous studies have failed to include butternut populations from New York, representing a significant gap in our understanding of butternut genetics, considering the number of trees and population that are present in New York. The objectives of this study used loci to determine: 1) genetic differences among sampled subpopulations of butternut; 2) demographic relationships among butternut trees; 3) correspondence of genetic data to life history traits, including differences among crown-class, diameter at breast height (DBH) cohort, bark type and vigor; and 4) comparative genetic diversity among and within habitats.

The first objective tests for population genetic divergence underlying our sampling scheme based on the null hypothesis of panmixia across sampling locations.
The second objective examines demographic relationships between subpopulations to test the null hypothesis that butternut trees demonstrate no correlation between geographic and genetic distance. The third objective evaluates the possible correlation of life history traits based on the null hypothesis that crown class does not correspond with genotype, and there is a heterozygosity deficiency between trees older and younger than the date of Oc-j introduction, butternut bark type does not correspond with genotypic patterns, and no relationship exists between genotype and vigor.

2.2 Materials and methods

2.2.1 Sampling

Leaf or bark tissue samples of suspected *J. cinerea* (n=237) were collected in July-August 2011 and June 2012 from 16 butternut clusters in Maine, New Hampshire, Vermont and New York. Each cluster is defined as a geographically separated aggregate of butternut trees. Pairwise Euclidean distances among sampling locations averaged 343 kilometers (Std.Dev. 228), and locations varied in growth habitat ranging from flood basin to mature upland forest. Our sampling design was based on a convenience sampling method relying on private and public landowners for information to locate trees. The 16 subpopulations were uniformly spread across the sampling area in order to minimize statistical error associated with sample clumping (Figure 1.1). Geographic coordinates (Table 2.1) were recorded, as well as habitat type, DBH, bark type, vigor, and crown class. Vigor, bark type, and crown class followed assessment parameters outlined in the Butternut Canker Disease Survey Protocol (Bergdhal et al, 2009).

The number of trees sampled in each location was dependent on availability and
time spent in the field. Small sample sizes can provide misleading data and were avoided when possible to reduce the probability of a Type 1 error occurring (Waples and Gaggiotti, 2006). A minimum of 10 trees was required to consider aggregated butternuts a cluster of sufficient size for sampling. Leaf tissue was removed from each tree, immediately placed in sealed plastic bags, stored in a cooler with ice until they could be transported to a 4°C refrigerator. Total genomic DNA was isolated from 10 mg of lyophilized plant tissue using the CTAB method (Doyle, 1987). Following extraction, DNA was resuspended in 200μL of 10mM Tris-HCL buffer, and the concentrations were estimated using a NanoDrop-2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). The DNA was aliquoted for standardizing to a concentration of 2.5 ng/μL and preserved at -20°C for short-term storage, the remaining DNA was frozen at -80°C for archiving.

2.2.2 Hybrid Analysis

To ensure our tissue samples were from pure butternuts and not hybrid butternuts, a hybrid diagnostic test developed by McCleary et al. (2009) was used. In summary, PCR was used to amplify cleaved amplified polymorphic (CAPs) sequence CPS02 (GenBank EU930860). The PCR reaction consisted of 1.5mM MgCl₂, 1x Green GoTaq buffer (Promega, Madison WI), 50μM of each dNTP, 0.7μM of each forward and reverse CPS02 primer, 1.0 ng of template DNA, and 2 units of Taq DNA polymerase. Total reaction volume was 10 μL per reaction and a negative control was run with all amplifications. PCR parameters followed an initial cycle of 2 min at 94°C for strand denaturation, followed by 30 cycles of denaturation (94°C, 30 s), primer annealing (57°C,
1 min) and a polymerase extension (72°C, 45 s). PCR finished with an extension (72°C, 10 min) and a rest period (4°C).

Aliquots of 10 uL CPS02 amplicons were subsequently digested using enzyme MSP I (New England BioLabs) and bovine serum albumin (BSA)(New England BioLabs) at 37°C for one hour. Digested CPS02 amplicons were electrophoresed through a 1.5% agarose gel stained with ethidium bromide, run at 75 volts for 45 minutes, and then visualized using Liminary FX (FOTODYNE Incorporated). Positive and negative controls were run with each reaction. Trees containing true *J. cinerea* chloroplast DNA display a single amplicon band of 332 base pairs (bp). In comparison, trees containing *J. alantifolia* chloroplast DNA have a cleaved amplicon of 235 bp and 97 bp in length (Figure 2.1)

Samples that contained *J. alantifolia* DNA and samples that could not be resolved in this hybrid test were removed from the study. Of the total 237 samples collected, thirty-one trees (13%) were verified to contain *J. ailantifolia* DNA. Two sampling locations contained only hybrid trees totaling 24, the remaining seven hybrids were detected growing among naturalized butternut stands (Table 3.1)

### 2.2.3 Microsatellite genotyping

Nine dinucleotide simple tandem repeats (STR) primers previously developed for *J. cinerea* (Ross-Davis and Woeste, 2007) were used to genotype sampled individuals (Table 2.2). Microsatellite amplifications were performed in 15 uL reactions containing 0.7 uM of each forward and reverse primer, 2 units of Taq DNA polymerase, 50 uM of each dNTP, 1x Green GoTaq buffer, 1.5mM MgCl₂, 100 ng BSA and 0.5 ng of template
DNA. PCR parameters included an initial cycle of 1 min at 94°C for strand denaturation was followed by a touchdown (5 cycles of denaturation (94°C, 30 sec), primer annealing (30 sec) at 66°C, 62°C, 58°C, 54°C, and 50°C, and polymerase extension (72°C, 30 sec)), then 34 cycles of denaturation (94°C, 30 sec), primer annealing (30 sec) at a primer-specific temperature, and polymerase extension (72°C, 45 sec). A final extension at 72°C for 10 min and a resting temperature of 4°C was included to minimize partial strands. Amplifications were performed in 96-well plates on an Express Gradient cycler (Denville Scientific) with positive and negative controls. Amplified samples were electrophoresed in a 1.5% agarose gel stained with ethidium bromide and visualized using Liminary FX to verify amplicon presence.

The forward primers were synthesized with either HEX or FAM fluorescent labels to allow for pool-plexing (grouped as follows: WGA147HEX; 221HEX; 204FAM; 256FAM and WGA004HEX; 082HEX; 090FAM; 148FAM). Verified amplicons were pool-plexed, diluted at a ratio of 1:10 with dH₂O, and submitted to the University of Wisconsin biotechnology center or the Hubbard Center for Genome Studies (HCGS) at the University of New Hampshire for analysis on an ABI 3130 Genetic Analyzer. Electrochromatogram were scored using GeneMapper v. 4.0 (Applied Biosystems Inc., Foster City, CA). Raw microsatellite data were reviewed manually to confirm correct identification and subsequently binned in Microsoft Excel. Bins in Excel allow for allele variation +/- one base pair from the true allele. Raw microsatellites that reported between bin ranges were re-analyzed for clarification. Individuals that could not be resolved were removed from the study.
2.2.4 Data analysis

Individuals that were missing >15% of their allelic data were removed from the data set. The culling of individuals with poor resolution left us with a total of 206 individuals from 16 populations. Many of methods used in this study could allow for some missing data. However, missing data can be particularly problematic for pairwise distance-based analyses such as AMOVA and Mantel tests because values used by computer programs to indicate missing data (ie. -9) are treated as identical. This results in perceived similarity between entities of missing data where no biological similarity may exist. To circumvent this problem, several programs can interpolate missing microsatellite data by inserting average genetic distances for each population level pairwise contrast. For instances where interpolation could not be applied, samples with missing data were removed from the dataset.

Subpopulations were tested for compliance to Hardy-Weinberg equilibrium, heterozygote deficiency and excess, and significance was estimated using the Markov chain Monte Carlo method with 1000 randomizations in GENEPOP v4.2 (Rousset, 2008). Tests for null alleles were performed with MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) with 1000 Monte Carlo simulations and a confidence interval of 95% adjusted by Bonferroni correction. Private alleles (those occurring in only a single subpopulation), alleles per loci (N_A), and allelic richness (R_A) were determined using GenALEX v6.5 (Peakall and Smouse, 2006).

To evaluate whether sampling locations displayed genetic structuring, unbiased Θ_{ST} measurements of Wright's F-statistics (F_IT, F_ST, F_IS) (Wright, 1931) expected heterozygosity and observed heterozygosity, and Shannon's diversity index (Shannon and Weaver, 1949) and their associated P-values were calculated using FSTAT v2.9.3 (Goudet,
Recently diverged populations are better resolved using estimates of $\Theta_{ST}$. Values that differ significantly from zero are used to reject the null hypothesis of panmixia (Balloux and Lugon-Moulin, 2002).

To further evaluate distinctive structuring among subpopulations, the program STRUCTURE v2.3.3 (Pritchard et al., 2000; Pritchard et al., 2003) was used. STRUCTURE uses a Bayesian-based algorithm to identify clusters of distinctive allele frequencies, regardless of their sampling origin. Genetic clustering was tested by specifying the number of potential populations ($K$), ranging from the null hypothesis of panmixia ($K=1$) through to the maximum number of sampled subpopulations ($K=16$). Twenty iterations of each $K$ were performed in STRUCTURE. This process was run twice with varying burn-in values of 100,000 and 300,000 and a Markov Chain Monte Carlo (MCMC) set to 100,000. These values were selected as a compromise between computational power and a stabilizing log alpha and Ln likelihood ($Ln(k)$). We examined consistency among the two burn-in replicates and the grouping patterns of individuals however only one is related in this paper. Optimal $K$-value was determined by the delta-$K$ likelihood evaluations from Evanno et al. (2005) as implemented in the program STRUCTURE HARVESTER (Earl and vonHoldt, 2012).

Isolation by distance was tested by a comparison of geographic and genetic distance. A Paired-Mantel test (Smouse and Long, 1992; Smouse et al., 1986) was implemented in GenAlEx to correspond pairwise orthodromic distance (haversine formula) to pairwise $R_{ST}$ and $\Theta_{ST}$ (Weir and Cockerham, 1984). Similar to $F_{ST}$, Slatkin's $R_{ST}$ summarizes the degree of differentiation between sub and total populations (Slatkin, 1995). The two measures differ in underlying assumptions on genetic mutation. Where
$F_{ST}$ assumes the infinite allele model (IAM), $R_{ST}$ assumes a stepwise mutation model (SMM). While the $R_{ST}$ is theoretically a more appropriate model for microsatellites, $F_{ST}$ appears to reflect actual differentiation more precisely. Mantel tests of 999 permutations contrasted $R_{ST}$ and $\Theta_{ST}$ to geographic distance and a third matrix of randomized data for significance. A spatial autocorrelation was performed in GenALEX using Weir and Cockerham (1984) $\Theta_{ST}$ and orthodromic pairwise matrices. Two spatial autocorrelation tests were run with even distance classes of size 25 with varying number of distance classes (10 and 25), 999 permutations were performed each with 1000 bootstraps. A self-assignment of individuals to sampling sites was tested in the Bayesian program GENECLASS2 (Piry et al., 2004) using a simulated population size of 10,000 individuals per site and a rejection level of 0.01 (Cornuet et al., 1999). We then compared the results of GENECLASS2 with the results of STRUCTURE and the pairwise genetic distance analyses.

To analyze the correspondence of genetic data to life history traits we calculated the percentage of variance and its significance by means of analysis of molecular variance (AMOVA) implemented in GenALEX. Various scenarios tested molecular variance among crown-classes, DBH cohort of 10 cm, bark type and vigor. An unconstrained approach employed the use of a $F_{ST}$ distance matrix (Sorensen) to conduct a non-metric multidimensional scaling (NMDS) to ordinate the relatedness between genetics and phenotype. Microsatellite alleles were treated as categorical variables, Sorensen distance matrix was run through 500 iterations in PC-ORD. For effective communication bark phenotype, vigor, habitat, epicormic count and canker number were all used as ordination overlays. PC-ORD was further used to conduct a cluster analysis. A Sorensen distance matrix was used to calculate a nearest neighbor linkage tree. Field
observations were used to guide interpretation.

Sampling methodology has a strong influence on the results of genetic analyses. Actual genetic structuring may be obscured by patterns of population division via sampling. GenAlEx was used to calculate F-statistics for each hypothesis for use in an AMOVA of within and among genetic variation for each hypothesized group. In addition to the AMOVA, Nei’s unbiased genetic distance (Nei, 1978) was calculated and used to perform a Principal Coordinate Analysis (PCoA). The procedure in GenAlEx is based on algorithms published by Orlóci (1978) and used a standardized-covariance method. Axes 1 through 3 were analyzed to test for panmictia.

2.3 Results

2.3.1 Genetic variation within subpopulations

Nine loci were initially amplified for the analysis of 227 butternut trees from 17 sampling sites across the northeastern USA (Table 1). Three of these loci (WGA148, WGA221 and WGA142) showed evidence of null alleles or had insufficient amplification for a majority of the samples. These loci, along with individuals of insufficient data coverage were removed from the study and were not included in further analyzes. The study was therefore based on 206 individuals at 6 loci. Overall the remaining loci were informative for use in structuring analysis as indicated by their relatively high $F_{ST}$ value (Table 2.2).

The number of alleles per locus ranged from 8 (WGA90) to 24 (WGA4) with the overall greatest variety of alleles occurring in sample sites ABC, BB, LAN, and MER (Tables 2.1). Sample sites with the lowest allelic richness occurred in Maine or in sites...
where the number of trees sampled was low. Heterozygosity (H_0) was highest towards the northeastern portion of butternuts range at sample site JP (H_0=0.850) and lowest towards the south-southwest portion of our sampling efforts in New York (JE=0.570, HF=0.583, PW=0.593). Half of the sampling sites displayed private alleles, the largest number of private alleles (N_pA) occurring in sample sites YER, MS, and LAN. The degree of relatedness within a population was relatively low with a range of 0.542 (GM) high to 0.042 (JW) low and a global mean of 0.144.

2.3.2 Genetic divergence among subpopulations

The majority of sample sites are genetically different from each other, rejecting the null hypothesis of panmixia (objective 1). Around half of the pairwise R ST values were significant, in contrast to the majority of F ST values reporting as significantly divergent (Table 2.3). Despite the lack of congruence of significant values, R ST and F ST pairwise matrices were not significantly different as indicated by a paired Mantel test (P=0.016; R^2=0.103). Sample site F ST values did not differ significantly for the following subpopulation comparisons: MS vs. JW, JP vs. JE, and PW vs. JE, JW, MS.

Genetic differences among subpopulations appeared to be independent of spatial isolation or geographic distance as indicated by a Mantel regression (P=0.230, R^2=0.0156) of genetic distance (Θ ST /1 - Θ ST) and geographical distance (km). Despite the slightly positive trend, Figure 2.2 demonstrates a lack of isolation-by-distance. Thus, some sampling sites that are geographically close are divergent (e.g. ABC and NOC) and some distant sites appear genetically similar (e.g. BF and JW). Both spatial autocorrelations confirmed results from the Mantel regression. High resolution distance
classes (10) and the larger depth of field (25) each revealed a non-significant correlation between site distance and divergence (Figure 2.3).

AMOVA testing revealed no significant relationships of butternut genetic structuring to crown class ($P=0.061$) or bark type ($P=0.243$), thus confirming the null hypothesis of no genetic relationship to crown class or bark type (Table 2.4). The relationship between genetic structure and DBH cohort was significant ($P=0.008$) indicating that tree age has some influence on genetic structuring, which was less pronounced than individuals among the cohort ($P=0.001$). Vigor class tested significant for all fixation indices with low $F_{ST}$ among vigor classes ($P=0.005$) and a more pronounced $F_{IT}$ value among individuals within a vigor class ($P=0.001$).

Bayesian STRUCTURE analysis identified $K=2$ population clusters that separated subpopulations around the Connecticut River valley from subpopulations both east and west of the Connecticut. The $K=6$ and $K=10$ scenarios were also justified by the likelihood method (Evanno et al., 2005) demonstrating a number of possible population subdivisions (Figure 2.4).

The Bayesian assignment test run in GENECLASS2 correctly self-assigned 32% of the individuals to their respective subpopulations (Table 2.5). The range of correctly assigned individuals varied from the highest at YER (84.6%) to the lowest at sites HM and GM (0%). Subpopulations with few samples appeared to have the lowest proportion of properly assigned individuals. Results from the GENECLASS2 analysis overall appeared to be similar to the clustering assignment from the program STRUCTURE (Figure 2.5). Principal coordinate analysis (PCoA) overlaid with assignment probabilities generated in structure reveal no real patterns of genetic structuring (Figure 2.6).
2.3.3 Genetic relation to life history

The stability of the NMDS ordination was assessed by the relative stress within a scree plot. A 2-dimensional solution provided sufficiently low stress in accordance with Clark's rule of thumb (Clarke and Ainsworth, 1993). The difference in stress between a 2-D (7.671) and a 3-D (4.196) solution did not warrant further investigation into a more complex ordination (Figure 2.7). NMDS and cluster dendrograms each revealed similar connections between phenotype and genotype. Crown class, DBH, canker number, and epicormic all showed genetic uniformity where no genotype was associated with any particular attributes. Strong genetic clustering was attributed with habitat in both cluster dendrogram (Figure 2.8) and NMDS ordination (Figure 2.9).

2.4 Discussion

2.4.1 Genetic diversity in northeastern butternut

Past studies have found relatively low genetic diversity in butternut populations of the northeast (Hoban et al., 2010; Morin et al., 2000; Ross-Davis et al., 2008), which may be the result of genetic bottlenecks due to population size fluctuations, or attributed to the marginality of sampling sites. Previous studies in plant populations have demonstrated a lower diversity in marginal populations (Lönn and Prentice, 2002) and butternut likely follows this gradient of ecological marginality at the periphery of its range. In the present study, all butternut sampling sites had similar observed heterozygosity (range=0.57-0.85, mean=0.67), lower than previously reported heterozygosity values for similar locations (range=0.789-0.842; Hoban et al. 2010 pop 17-20) (Ontario pop Ho=0.83; Ross-Davis et al. 2008).
The genetic diversity study performed by Hoban et al. (2010) contained four populations (17-20) that were clustered geographically in the state of Vermont, near the middle of our sampled subpopulations. Their mean expected heterozygosity was 0.822 and ours was 0.670, their allelic richness was 7.51 and ours 5.35. These population estimates differ to the extent that caution should be exercised when comparing results. Differences can be attributed to a number of factors including the location of sampling, microsatellites used, and variation in sampling size (n=82 vs. n=206). The dense sampling scheme and large sample size in this present study (n=206) likely provides a more accurate estimation of population differentiation for northeastern butternut. The present study provides an estimation of northeastern population differentiation at a spatial resolution more fine than previous studies. Differences in measurements of identity by descent (IBD) may differ due to the STR markers used.

The ability to identify populations is likely compromised by the sampling approach. An ad hoc approach to sampling the continuous range of an organism could lead to erroneous assumptions on processes restricting gene flow. For a population where gene flow is restricted by isolation by distance, discrete populations can be incorrectly identified and management policy inappropriately applied (Schwartz and McKelvey, 2008). Northeastern butternut do not display IBD (Figure 2.3) allowing for more appropriate predictions on processes of gene flow, however the convenience sampling approach used in this present study may provide misleading predictions of K populations (Schwartz and McKelvey, 2008).

Due to the lack of genetic data for butternut in northeast, it is reasonable to compare genetic data of butternut to other broad-leaf tree species (Pautasso, 2009). Using
some of the same microsatellites as used in the present study, Victory et al. (2006) found the congener species *J. nigra* to have a mean heterozygosity of 0.807. Victory's results, along with the results from other broad-leaf studies on *Fraxinus excelsior* (H₀=0.82) and *Populus nigra* (H₀=0.74) (Heuertz et al., 2004; Smulders et al., 2008) exemplify the low levels of heterozygosity detected in butternut. The present study sampled from subpopulations in the periphery of butternuts natural range. Gapare et al. (2007) found a fine-scale study of small populations in the peripheral would only account for 68-76% of the maximum expected heterozygosity for the whole range. If our results were to account for only 76% of the range-wide heterozygosity, then our results align more closely with range-wide heterozygosity calculations from other publications.

A population that is divided into isolated subpopulations will contain less heterozygosity than if the population was undivided. This is a product of inbreeding and drift in small populations. Six of our sample sites were significantly deficient (p<0.05) in heterozygotes, four of which are found along the Connecticut River, which creates the border between New Hampshire and Vermont. When tested with Bayesian statistics, these same four populations (BB, ABC, LAN, YER) had the highest rates of self-assignment (Table 5). Founder effects and inbreeding acting on these four subpopulations generally lead to subpopulations with allele frequencies that are different from the larger population. Also, these subpopulations are theoretically smaller in size than the larger population as discovered from their heterozygosity; there will be greater sampling error in these small groups than there would be in a larger undifferentiated population. Hence, genetic drift will push these smaller demes toward different allele frequencies and allele fixation more quickly than would take place in a larger undifferentiated population. As
expected, the inbreeding coefficient ($F_{IS}$) for these four populations is higher than the average inbreeding coefficient for the entire population. Sampling methodology whereby a single population was sampled as separate populations may have contributed to the observed deficiency in heterozygosity.

Alternative to heterozygote deficiency, subpopulations were tested for heterozygote excess. Only one subpopulation (JP) was observed to have a significant excess ($P=0.019$) in heterozygotes relative to Hardy-Weinberg expectations. Heterozygote excess is less common than a deficiency in natural populations and is therefore not fully explored. In general there are two major explanations for an excess in heterozygotes: 1) overdominant selection favoring heterozygotes (Li et al., 1998); and 2) disassortative mating (O’Malley and Bawa, 1987). Disassortative mating in walnuts was first reported in 1982 relating to the heterodichogamous nature of *J. hindsii* and *J. regia* (Gleeson, 1982). *Juglans* spp. produce male and female flowers at separate times to prevent inbreeding, thus disassortative mating is a mechanism to maintain outcrossing. The result of this mechanism can be observed as an excess in heterozygotes.

Based on breeding mechanisms, the norm that should be observed for all butternut subpopulations is an excess in heterozygotes. Interestingly, the majority of populations tested positive for a deficiency in heterozygosity. An explanation for this phenomenon is difficult to place relying on microsatellites and observational data alone. However, northerly remote features of the JP population leave a possibility that *Oc-j* was only recently introduced to the area. All of the trees at the JP location are large enough to have developed well before the first reports of *Oc-j* in the area. Butternut canker also had a low observable impact on the JP subpopulation. It is possible that trees of more southerly
locations have been under the stress from Oc-\textit{j} thus reducing the numbers of breeding individuals and the possibility for disassortative mating. Alternative explanations to the observed excess in heterozygosity could be attributed to a sampling artifact based on small sample size. Could the imminent inbreeding depression observed in the heterozygote deficient populations be the result of population decline due to \textit{Oc-j}? A greater number of both heavily diseased and less effected populations would need to be sampled and compared to determine this potential phenomenon.

We found no significant correspondence between genetic diversity and geographic distances in butternut subpopulations. This result was verified by a number of analytical approaches and can be explained by dispersal method of butternut. Butternut is a wind-pollinated species. Other studies of wind pollinated forest trees have demonstrated the ability of pollen to travel distances over 19 km (Ward et al., 2005). The maximum range of viable butternut pollen has not yet been reported but likely has influenced our ability to detect genetic structuring due to long distance dispersal.

The heterozygosity deficiency observed in butternut of the northeast is the result of a variety of factors. A combination of range periphery and population decline is likely the two largest factors contributing to small populations and heterozygosity deficiency. These natural factors are uncontrollable. Management policy should be oriented to maintaining the present genetic variation for both upland and riparian butternut stands. This can be achieved by ensuring sufficient gene dispersal between increasingly isolated butternut stands.
Figure 2.1. Chloroplast CPS02 marker fragments for *Juglans cinerea* (lanes 3-6) and *J. ailantifolia* hybrid. True butternut amplicon expected at 332 bp, whereas hybrid bands expected at 235 bp and 97 bp. Amplicons visualized on 1.5% agarose gel stained with ethidium bromide.
Figure 2.2 A Mantel regression comparing population geographic distance (y-axis km) to pairwise linearized $\theta_{st}$ distance matrix (x-axis). Isolation by distance is not apparent with a $p$-value of 0.23
Figure 2.3. Spatial structure analysis using distance classes of 25 (x-axis) and FST values along the y-axis. Significant correlation ($p=0.002$) of genetic uniformity across distance classes calculated from 999 permutations and 1000 bootstraps.
Figure 2.4. Structure bar plot of Q-values for populations K=2 (a), K=6 (b), and K=10 (c). Each bar relates to probability of assignment for individuals to a putative population. DeltaK calculated following Evanno’s method (2005) outputs likelihood of each K-value (d)
Figure 2.5. The Bayesian program STRUCTURE used to identify posterior possibilities for a subdivision of K=2(a), K=6(b), and K=10(c) populations. Assignment probabilities for each genotype are overlaid on respective sampling location.
Figure 2.6. Principal coordinate analysis of sub-populations using as a distance measure. Population assignment for K=2 calculated in STRUCTURE is overlaid for comparison of Bayesian and \( F_{ST} \) statistics.
Figure 2.7. Scree plot displaying stress used to determine dimensionality of NMDS ordination.
Figure 2.8. Cluster dendrogram based on Sorensen genetic distances displayed as nearest neighbor linkage tree. Individual trees are colored by habitat
Figure 2.9. NMDS ordination based on Sorensen genetic distances color coded by habitat type. Blue dots represent the loading of each locus.
TABLE 2.1. SAMPLING LOCATIONS AND GENETIC CHARACTERISTICS

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>N</th>
<th>H₀</th>
<th>Hₑ</th>
<th>uHₑ</th>
<th>Fₛₜ,µ</th>
<th>Fᵢₛ</th>
<th>Heterozygote</th>
<th>Rₐ</th>
<th>Nₐ</th>
<th>Nₚₐ</th>
<th>Pₚₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>44.654</td>
<td>-71.565</td>
<td>20</td>
<td>0.604</td>
<td>0.744</td>
<td>0.769</td>
<td>0.091</td>
<td>0.239</td>
<td>Deficient</td>
<td>6.833</td>
<td>48</td>
<td>1</td>
<td>0.021</td>
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<tr>
<td>BB</td>
<td>44.046</td>
<td>-72.064</td>
<td>22</td>
<td>0.595</td>
<td>0.691</td>
<td>0.731</td>
<td>0.078</td>
<td>0.211</td>
<td>Deficient</td>
<td>6.833</td>
<td>53</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>LAN</td>
<td>44.475</td>
<td>-71.622</td>
<td>25</td>
<td>0.672</td>
<td>0.687</td>
<td>0.717</td>
<td>0.093</td>
<td>0.091</td>
<td>Deficient</td>
<td>6.833</td>
<td>49</td>
<td>3</td>
<td>0.061</td>
</tr>
<tr>
<td>JE</td>
<td>43.222</td>
<td>-76.608</td>
<td>16</td>
<td>0.570</td>
<td>0.718</td>
<td>0.750</td>
<td>0.062</td>
<td>0.253</td>
<td>Deficient</td>
<td>6.500</td>
<td>45</td>
<td>0</td>
<td>0.000</td>
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<tr>
<td>PW</td>
<td>42.719</td>
<td>-74.110</td>
<td>11</td>
<td>0.593</td>
<td>0.681</td>
<td>0.725</td>
<td>0.064</td>
<td>0.183</td>
<td>Deficient</td>
<td>5.500</td>
<td>38</td>
<td>0</td>
<td>0.000</td>
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<tr>
<td>YER</td>
<td>43.519</td>
<td>-72.296</td>
<td>14</td>
<td>0.764</td>
<td>0.691</td>
<td>0.735</td>
<td>0.121</td>
<td>0.183</td>
<td>Deficient</td>
<td>5.333</td>
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<td>3</td>
<td>0.075</td>
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<td>GM</td>
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<td>-72.929</td>
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<td>0.500</td>
<td>0.667</td>
<td>0.128</td>
<td>0.542</td>
<td>Deficient</td>
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<td>KL</td>
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<td>-73.779</td>
<td>11</td>
<td>0.619</td>
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<td>0.647</td>
<td>0.121</td>
<td>0.081</td>
<td>Deficient</td>
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<tr>
<td>MSC</td>
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<td>-75.633</td>
<td>13</td>
<td>0.690</td>
<td>0.756</td>
<td>0.804</td>
<td>0.076</td>
<td>0.130</td>
<td>Deficient</td>
<td>6.167</td>
<td>43</td>
<td>2</td>
<td>0.047</td>
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<tr>
<td>HF</td>
<td>42.670</td>
<td>-73.650</td>
<td>10</td>
<td>0.583</td>
<td>0.585</td>
<td>0.645</td>
<td>0.130</td>
<td>0.201</td>
<td>Deficient</td>
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<td>26</td>
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<td>MS</td>
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<td>0.740</td>
<td>0.779</td>
<td>0.073</td>
<td>0.157</td>
<td>Deficient</td>
<td>6.167</td>
<td>42</td>
<td>3</td>
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<td>NOC</td>
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<td>0.608</td>
<td>0.678</td>
<td>0.150</td>
<td>0.138</td>
<td>Deficient</td>
<td>3.833</td>
<td>29</td>
<td>0</td>
<td>0.000</td>
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<td>43.657</td>
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<td>20</td>
<td>0.771</td>
<td>0.739</td>
<td>0.761</td>
<td>0.077</td>
<td>0.062</td>
<td>Deficient</td>
<td>6.833</td>
<td>49</td>
<td>2</td>
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<td>JW</td>
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<td>0.736</td>
<td>0.706</td>
<td>0.757</td>
<td>0.061</td>
<td>0.042</td>
<td>Deficient</td>
<td>6.000</td>
<td>40</td>
<td>1</td>
<td>0.025</td>
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<tr>
<td>BF</td>
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<td>-69.006</td>
<td>7</td>
<td>0.667</td>
<td>0.659</td>
<td>0.723</td>
<td>0.092</td>
<td>0.098</td>
<td>Deficient</td>
<td>4.667</td>
<td>31</td>
<td>1</td>
<td>0.032</td>
</tr>
<tr>
<td>JP</td>
<td>44.510</td>
<td>-70.519</td>
<td>5</td>
<td>0.850</td>
<td>0.612</td>
<td>0.705</td>
<td>0.105</td>
<td>-0.299</td>
<td>Excess</td>
<td>3.333</td>
<td>22</td>
<td>0</td>
<td>0.000</td>
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<tr>
<td>Total</td>
<td>206</td>
<td></td>
<td></td>
<td>0.670</td>
<td>0.670</td>
<td>0.084</td>
<td>0.144</td>
<td>5.354</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Sample locations are in degrees latitude north and longitude west. Sample size (N), observed heterozygosity (H₀), expected heterozygosity (Hₑ), unbiased expected heterozygosity (uHₑ), allele richness (Rₐ), are averaged across all loci. Nₐ indicates number of alleles, Nₚₐ the number of private alleles, and Pₚₐ the proportion of private alleles. Significance of heterozygosity deficiency or excess is indicated in bold (P<0.05).

µ – Mean pairwise Fₛₜ
## TABLE 2.2. DESCRIPTIVE STATISTICS FOR MICROSATELLITES

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<thead>
<tr>
<th>Locus</th>
<th>NA</th>
<th>Size Range (bp)</th>
<th>$F_{IT}$</th>
<th>$F_{ST}$</th>
<th>$F_{IS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGA90</td>
<td>8</td>
<td>126 - 142</td>
<td>-0.002</td>
<td>0.069</td>
<td>-0.076</td>
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<tr>
<td>WGA4</td>
<td>24</td>
<td>225 - 273</td>
<td>0.169</td>
<td>0.073</td>
<td>0.104</td>
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<td>WGA82</td>
<td>15</td>
<td>153 - 181</td>
<td>0.178</td>
<td>0.075</td>
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<td>WGA256</td>
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<td>206 - 242</td>
<td>0.199</td>
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<td>WGA204</td>
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<td>172 - 196</td>
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<td>0.098</td>
<td>-0.013</td>
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<td>WGA147</td>
<td>9</td>
<td>174 - 200</td>
<td>0.358</td>
<td>0.068</td>
<td>0.311</td>
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<tr>
<td>Mean</td>
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<td></td>
<td>0.168</td>
<td>0.077</td>
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</table>
TABLE 2.3. COMPARISON OF PAIRWISE F$\text{ST}$ AND R$\text{ST}$ VALUES

<table>
<thead>
<tr>
<th>Location</th>
<th>ABC</th>
<th>BB</th>
<th>BF</th>
<th>GM</th>
<th>HF</th>
<th>JE</th>
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R$\text{ST}$ values below diagonal and F$\text{ST}$ values above diagonal. Non-significant pairwise values ($P>0.05$) indicated in bold.
TABLE 2.4. ASSOCIATION OF GENETICS TO BROAD GROWTH CHARACTERISTICS

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<th>Hypothesis 3 - Crown Class</th>
<th>Percent variation</th>
<th>Fixation index</th>
<th>Significance (p-value)</th>
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<td>Among the 4 Crown Classes</td>
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<th>Hypothesis 4 - DBH Cohort</th>
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<th>Hypothesis 5 - Bark Type</th>
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<table>
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Significance indicated by bold (P<0.05)
### TABLE 2.5. RESULTS FROM BAYESIAN SELF-_ASSIGNMENT

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Results from Bayesian self-assignment program GENECLASS2, numbers in highlighted diagonal represent individuals correctly assigned to the population from which they were sampled.
3.1 Introduction

3.1.1 Juglans cinerea

*Juglans cinerea*, known as the butternut tree or white walnut is an economically and ecologically important member of the eastern deciduous forest. Native to eastern North America the range of butternut stretches from southern New Brunswick and Quebec west to Minnesota and as far south and southwest as Georgia and Missouri. This medium sized tree is not abundant in forests, historically contributing 1-3% arboreal pollen (Delcourt, 1979) and are usually found as small stands in association with other mixed hardwoods (Schultz, 2003). Butternut is a relatively short lived species reaching maturity at 15-20 years and rarely exceeding 75 years in age (Ostry et al., 2003). A wind pollinated species, butternut produce significant seed crops every 2-3 years with lighter yields during interim years (Rink, 1990). The heavy seed requires dispersal by gravity, water, or scavengers and may not survive adjacent to the parent tree due to chemical inhibition and resource competition. *Juglans* species exude a naphthoquinone that is negatively allelopathic even to butternut seedlings (Hartman et al., 2000). This chemical inhibition, combined with butternut's shade intolerance, seed predation by animals, and narrow range of suitable growing sites make sapling recruitment infrequent.

Further compounding the effects of low recruitment, butternut is under threat of extinction by an exotic fungus. A notable disease on butternut was first reported in Wisconsin 1967 (Renlund, 1971), the disease is widely known as butternut canker caused
by the ascomycete *Ophiognomonia clavigignenti-juglandacearum* Broders and Boland.

Today *Oc-j* threatens the entire range of butternut responsible for mortality rates approaching 80% (Ostry et al., 2003). *Oc-j* can infect butternut trees of all ages often killing saplings more rapidly than mature trees.

Initially, butternut canker was not reported in New England and New York (Anderson and LaMadelaine, 1978), however by 1982 *Oc-j* was present in the northeast (Kostichka, 1982). More recent surveys in the northeast reveal a disease incidence rate of nearly 100% and a mortality rate of 25% (Bergdahl, 2009). It still remains unclear how *Oc-j* spread throughout butternut’s range so rapidly, but coleopteran vectors (Halik and Bergdahl, 2002) combined with anthropogenic mediated jump dispersal via infected seed (Innes and Rainville, 1996) and multiple introductions (Broders et al., 2012) may have all contributed to the rapid dispersal.

Resistance to *Oc-j* is essential for the long-term survival of butternut (Michler et al., 2006). While some butternut trees appear to be affected by butternut canker to a lesser degree than others, no specific mechanism of resistance has been identified. Understanding the parameters of butternut canker in a natural setting can help guide conservation policy and perhaps elucidate factors that contribute to *Oc-j* resistance. Furthermore, monitoring butternut recruitment along with the proportion of hybridization will provide insight on invasion biology.

### 3.1.2 Genetic invasion

The butternut tree, a predominantly outcrossing species, has the ability to naturally hybridize with at least two other exotic congeners, Japanese walnut (*J.*
ailantifolia Carr.) and English walnut (J. regia L.), which were introduced to North America during the mid-19th century for use as ornamentals and for nut production (Ostry and Moore, 2007). These exotic Juglans species have naturally hybridized with butternut since their introduction (Ostry and Woeste, 2004). It is common for woody taxa from Asia to successfully hybridize with its North American sister taxa (Wen, 1999), however, the hybridization of disjunct species generally results in extrinsically and intrinsically unfit progeny (Mayr, 1992). Whether or not genetic invasion becomes a concern depends on the fitness and fecundity of successful hybrids. With increased hybrid fitness, amalgamation of both parental genotypes is expected (Rieseberg, 1997).

Juglans ailantifolia and butternut hybrids (J. x bixby) are reported to be less susceptible to canker disease when inoculated with Oc-j when compared to true butternuts (Orchard et al., 1982). Furthermore, it has been repeatedly observed that butternut hybrids and other Juglans species in natural settings are less affected by Oc-j than true butternuts (Ostry, 1997). Recent evidence indicate that butternut hybrids show greater resistance to natural infection by Oc-j, when compared to pure butternuts (McKenna et al., 2011). The mechanisms of this resistance are unclear, though theory indicates a coevolution between Oc-j and Japanese walnut (Furnier et al., 1999). When a pathogen and host disease-system coevolves, there is a constant evolution of resistance and avirulence genes developed by both host and pathogen (Hammond-Kosack and Jones, 1997). This is referred to as the "evolutionary arms race", which explains why a manageable disease may become an epidemic out of its natural range. Coevolution between Japanese walnut and Oc-j is believed to be a contributing reason for reduced symptoms of butternut canker on Japanese walnuts. Regardless of the mechanisms for resistance, butternut hybrids that
are less susceptible to butternut canker will have a higher fitness and increased probability of survival and reproduction.

Japanese walnuts and related hybrids are generally used as ornamentals or for nut crop production and are usually restricted to fragmented semi-rural landscapes (Ostry and Woeste, 2004). The rate of butternut hybridization is still unknown due to limited range-wide monitoring and difficulties associated with complex hybrid backcrossing. Rapid genetic invasion is suspected not to have occurred as Japanese walnuts were introduced to North America from Japan around c. 1870 (Manning, 1978) and pure butternuts are still present in greater abundance (Zhao and Woeste, 2010). However, the vigorous growth of hybrid butternuts and an increased resistance to butternut canker may tip the balance of gene flow in favor of Japanese walnut and butternut crosses. If this were true, we would see the gradual integration of Japanese walnut genes into the gene pool of butternut trees. *Juglans* species that were once geographically separate now interbreed and produce vigorous hybrids potentially capable of out-competing either parental species (Hoban et al., 2009). Cultivated hybrids of Japanese walnut and butternut are both vigorous and prolific to the extent that concern of genetic invasion has already been raised (Ostry and Woeste, 2004). From a conservation standpoint, genetic invasion may result in the muddying of butternut's gene pool and the eventual loss of a defined species. For butternut, as in case with the American chestnut (Diskin et al., 2006), hybridization may provide the only means of persistence.

This unique situation allows us to understand the consequences of an exotic fungus that has tipped the balance of gene flow in favor of hybrids, where it was previously uncertain whether hybrids could persist in a natural setting.
3.1.3 Objectives

The extent of recruitment and the health of butternut trees have been monitored for several places in the US, notably areas surrounding Wisconsin, Tennessee, Connecticut, and Vermont. There is, however, a lack of data on the health of butternut and the hybrid status of butternut in New York. With reportedly low recruitment frequency, understanding characteristics that allow butternut to grow until seed-bearing maturity is important for future restoration projects. The overall goal of this project is to aid butternut restoration by providing statistical information on the health, ecology and recruitment of butternuts. Objective 1 entails locating butternut subpopulations (N>10) and taking general health, ecological, and growth parameters for individuals within a subpopulation. The data collected for this objective will help identify the health status of butternut in the northeast, level of recruitment, and potentially identify sources of resistance. Objective 2 aims to quantify the level of butternut hybridization in the Northeast to evaluate the threat of genetic invasion by conspecifics.

The two objectives aim to convey information on the presence and health of butternut in the northeast. Observational results from previous butternut reconnaissance have suggested that butternut with deep-fissured bark appear to be more resistant to Oc-j than do butternut with shallow fissured bark. In this study we test the null hypothesis (a) that butternut trees of various bark phenotypes are equally affected by butternut canker. Additional hypotheses tested include (b) trees growing in upland habitat vary from riparian trees by the magnitude of disease impact, (c) trees that were present prior to Oc-j introduction display a similar response to Oc-j as compared to trees establishing post Oc-j introduction. Objective 2 evaluates the threat of genetic invasion by hybridization by testing the hypothesis (d) that hybrid trees demonstrate increased resistance to Oc-j.
3.2 Methods

3.2.1 Study location

The sampling method used is an integral part of any experimental design. We relied on a subjective convenience sampling method. Convenience sampling is a method of data abstraction whereupon the observer selects samples most easily accessible. Convenience sampling was a necessary part of our data acquisition due to the relatively low abundance and unpredictable evenness of butternut stands. Models predicting the location of butternut remain unreliable (Thompson et al., 2006) and combing forests for butternut stands is costly and time consuming. The intensity and coverage of field sampling efforts were constrained by the duration of the project period. For this study, field work occurred during the months of August 2011 and June 2012. Field work entailed locating, observing, and sampling butternut trees. This process was guided by auxiliary information regarding butternut location and population sizes. Butternut sampling locations were determined in four ways; (I) using GPS coordinates of butternuts that have been sampled for other projects, (II) census data from private and public landowners, (III) sampling in areas suspected of butternut growth, and (IV) from information provided by local forester and state park personnel. The 2011 field season focused mainly on sampling methods I, III and IV, whereas the 2012 field season relied heavily on sampling method II. Although convenience sampling can bias the outcome of a study, there is little reason to believe our samples would differ from randomly chosen individuals in the same population. Furthermore, our sampling method allowed for the sampling of more butternut trees when compared to serendipitous encounter. The subjective nature of convenience sampling reduces the ability to calculate ecological inferences on relative abundance and distribution of butternut, although the use of genetic
STR's provides the level of population differentiation addressed in this study. The selection of sites for sampling was more dependent on the number of reported trees in a subpopulation rather than the geographic location of the subpopulation. The criteria for site selection were flexible. Site selection was based on the number of reported trees at a location. Sites were chosen that reportedly had butternut stands of greater than ten individuals. This selection criterion was influenced more by the genetic analyses portion of this project rather than the life history portion.

A number of factors predispose the habitat type most often sampled. A majority of the sampled trees were located in the riparian zones of long established farm properties. Most forests in the northeastern U.S.A. have been logged, burned and farmed prior to 1900 (Williams, 1992). For this reason, forested sites represent the conditions of the last 100 years. Current hardwood forest management in the northeast favors disturbance suppression, limiting the amount of natural upland butternut regeneration due to uninterrupted shade canopies. Butternut growth habitat is quite similar to that of other hardwood species like maple, birch and ash. These longer-lived tree species will suppress the recruitment of butternut, and without disturbance butternut is ebbed from forest stands. Small farms and riparian zones are suitable for butternut recruitment due to the high levels of disturbance. Agricultural practices suppress the establishment of forests by maintaining open fields. Field borders and fencerows provide sufficient light to allow the establishment and long-term growth requirements necessary for butternut to flourish. Butternuts along agricultural boundaries provide farms a nut crop each year (Ostry and Pijut, 2000). In addition to growing well on farm properties, butternuts readily establish in floodplains and riparian zones. Flood plains provide enough disturbances to allow the
establishment of grasses and fast growing woody plants. Flood plains provide open canopies and a consistent source of water, necessary for rapid growth in butternut. Furthermore, nearby flowing water is an excellent vector for butternut's large and heavy seed. At maturity, the butternut seed weighs 750 g and is 3-6 cm in diameter (Hewitt, 1998). Floodplains and farm properties represent a majority of the sampling sites we visited for sampling.

Sampling efforts in August 2011 were limited to New Hampshire and focused around the Connecticut River basin and adjacent properties. Butternut stands were identified with the help of state foresters. In total, four locations along the Connecticut River were sampled Bedell Bridge, Lancaster, Stratford, and the Yatsevitch forest. In addition to these population, one population (Meredith) located in interior New Hampshire, was sampled. Locations were sampled during the month of August and locations were spread amongst three sampling trips ranging from one day for nearby locations, to one week for further locations.

Sampling efforts in June of 2012 totaled twelve locations across Maine, Vermont, and New York. Butternut stands were located as far west as Dunkirk NY, as far east as Belfast ME, and as far north as Mooers Forks NY - near the Canadian Border. The vast majority of sampling locations were located on privately owned land. Owners were contacted with the help of Cornell Cooperative extension and the New York State Forest Owners Association. All butternut sampling for the 2012 field season occurred over the span of two weeks in June. Sampling sites varied in habitat type and surrounding vegetation. On occasion sample sites contained more than twelve butternut trees. When this occurred, only the first twelve trees encountered would be included in this study.
Our sampling scheme anticipated 10 trees sufficient to represent a subpopulation. Sampling resources dedicated to a larger number of subpopulations rather than larger subpopulations provide greater representation of butternut’s northeastern range. Combining the two sampling seasons, pairwise Euclidean distances between 16 sampling locations averaged 343 kilometers, and locations varied in growth habitat ranging from flood basin to mature upland forest.

Several sites visited had butternut trees, whereupon inspection was deemed to be hybrid butternut trees. Field experience and guidelines provided by Amy Ross-Davis et al. (2008) aided the field identification of hybrid butternut trees. If the butternuts at a sample site were not butternut, or were clearly hybrid butternuts, than the site was avoided and not sampled. For trees where it was uncertain whether trees were of hybrid nature, tissue samples were obtained for molecular analyses. Often within a butternut stand, dead butternut trees would be encountered as downed trees or snags. Without live tissue it would not be possible to test the hybrid status of a dead tree. To avoid confusion in our dataset, dead trees were not included in the dataset because our experimental design did not compensate for factors including time since death or cause of death.

3.2.2 Survivorship and health

Factors like allelopathy and light requirement predispose butternuts to grow in sparse stands. For this reason it is difficult to locate butternut trees due to their sparse growth pattern. The weight of butternut seed does not allow for long distance travel and a located butternut tree is usually indicative of other butternuts growing nearby. This grouping pattern allowed for the sampling of butternut clusters. It is important to note
that clusters of butternut do not necessarily imply populations of butternut, but rather an aggregate of related trees in an area. It would also be safe to assume that trees sampled within an aggregate are subject to similar climactic and ecological patterns. Observations were recorded of each tree to assess how environmental factors play a role in butternut survivorship. Statistical parameters recorded from each tree were: GPS coordinate and elevation, diameter at breast height (DBH), a rating of tree vigor, habitat type, number of cankers, level of crown dieback and the number of epicormic growths. These parameters are similar to the parameters chosen by Ostry et al. (1994) to designate healthy butternut trees. Ostry et al suggest a 70-20-50 rule where trees with more than 70% live crown and less than 20% of the circumference affected by cankers, should be considered healthy. Trees with 50% live crown and no cankers are also considered healthy. These guidelines have been used by other butternut researchers (Parks et al., 2011) and are used to establish a baseline of general health, age and geographic location. Other measures of health were recorded and are outlined below.

The diameter at breast height (DBH) can be used as an estimate of tree age according to models developed for *J. nigra* (Frelich, 1992). In North America, DBH is located on the trunk of a tree 1.3 meters off the ground. For trees growing on a slope, DBH is measured from the highest point that ground contacts the base of the tree. A measuring tape was used to determine the circumference of the tree from which the diameter was calculated. Estimating tree age using DBH has its inherent flaws. Many factors including competition, access to nutrients, genetic disposition and season length can influence the rate of growth and in turn the girth of a tree. For this reason, DBH provided only an estimate of tree age.
An ordinal rating scale for tree vigor was used as an estimation of tree health. Parameters for vigor have been classified into four general categories; (1) dead, the tree has clearly not experienced any growth within the last season; (2) nearly dead, some life remains though the tree is clearly desiccating; (3) fairly vigorous, while there is some evidence of desiccation the tree is predominately thriving; and (4) vigorous, the tree is clearly thriving with little or no evidence of desiccation. While estimation of vigor is subjective, the broad nature of these parameters increased the likelihood of consistent assignment. Other parameters measuring general tree health, number of cankers, crown dieback and epicormic growth, was used to reinforce estimations of vigor. Canker number is categorized by activity and location on the trunk that was used to complement estimations of vigor. Cankers were counted on trunk segments between 0.3-2.7 m, both active and healed cankers were counted and used as a ratio ((# unhealed cankers/total canker #)*# unhealed cankers). Crown dieback was estimated by visual observation and categorized according to foliage abundance on a scale of 1 to 4. The crown dieback scale was an ordinal scale where canopies were rated as (1) minimal dieback (<10%), (2) moderate dieback (10-50%), (3) heavy dieback (50-80%), and (4) severe dieback (>80%). Crown dieback is related to the number of epicormics in that the loss of canopy in butternut trees results in the shift of hormonal balance to favor the growth of epicormic branches. The development of epicormics is evidence of a butternuts attempt to re-establish photosynthetic growth. The number of epicormics is used as a supplemental estimator of canopy health and ultimately to complement ratings of tree vigor.

The sampling of butternut trees can be difficult due to their limited numbers and dispersed growth pattern. Butternuts generally favor riparian zones for a habitat. To
ameliorate bias from sampling butternut trees exclusively in watersheds, every effort was
made to collect data and sample trees growing in upland locations. Data recording
included a category assigning samples to either upland or riparian habitats. Determining
whether a butternut is categorized as upland or riparian depended on the vicinity to, and
the relative elevation from a water source. Trees that were located within 50 meters of a
permanent body of water or within a floodplain were designated as riparian. All other
butternuts that did not meet these criteria were categorized as upland.

3.2.3 Butternut dendrochronology

A simple statistical correlation between tree age and DBH allows for the
estimation of DBH as an estimator of tree age. Frelich (1992) determined a highly
significant correlation between the age of black walnut and its DBH. The age of a black
walnut tree was estimated with the non-linear model \( DBH = 32.29(1 - e^{(-0.0194)(age)^{1.260}}) \) to predict tree age using DBH. This equation is relatively accurate
and has an \( R^2 \) value of 0.903. Butternut canker was not reported in New Hampshire and
Vermont in 1978 (Anderson and LaMadelaine, 1978), but was later reported to be spread
throughout the northeastern US by 1994 (Ostry et al., 1994). These two reports provide a
16-year range in which we can expect the establishment of butternut canker in the
northeast. Regenerating trees from that time period are expected to have a range in DBH
between 17 and 32 cm. This range served as a guideline for determining trees that have
established pre- and post-\( Oc-j \) invasion. Tree cores were taken from eight butternut trees
to test how closely Frelich's model of black walnut fit to butternut.
3.2.4 Vegetation sampling

A small amount of plant tissue was removed from sampled trees. For trees with unreachable canopies, a small section of cambium was excised from under the bark. This tissue excision was performed with a pocket knife and was no larger than 3 cm\(^2\). If a canker site existed on the trunk, cambium excision occurred on the margin of the canker for downstream culturing of \(Oc-j\). Cambium is preferred to bark because it contains living plant cells resulting in higher quality DNA extractions. For the majority of trees, an arborist sling-shot was used to retrieve a sample of leaf tissue from the canopy. Obtaining leaf samples is preferred to cambium excision due to the possibility of inoculating a fresh wound in the cambium and presenting a higher risk of infection than the scar produced from leaf removal. Furthermore DNA extraction from leaves resulted in larger quantities of higher quality DNA. Plant tissue removed from trees was organized into individual plastic bags which were placed on ice in a cooler until they could be stored in the 4°C refrigerator and lyophilized. Each bag is marked with an identification number associated with the trees statistical data.

3.2.5 Hybrid Analysis

To ensure our tissue samples were from pure butternuts and not hybrid butternuts, a hybrid diagnostic assay developed by McCleary et al. (2009). In summary, PCR was used to amplify cleaved amplified polymorphic sequence CPS02 (GenBank EU930860). The PCR reaction consisted of 1.5mM MgCl\(_2\), 1x Green GoTaq buffer (Promega, Madison WI), 50\(\mu\)M of each dNTP, 0.7\(\mu\)M of each forward and reverse CPS02 primer, 1.0 ng of template DNA, and 2 units of Taq DNA polymerase. PCR parameters followed
an initial cycle of 2 min at 94°C for strand denaturation, followed by 30 cycles of denaturation (94°C, 30 s), primer annealing (57°C, 1 min) and a polymerase extension (72°C, 45 s). PCR finished with an extension (72°C, 10 min) and a rest period (4°C). Total reaction volume was 10uL per reaction and a negative control was run with each amplification.

Aliquots of 10uL of CPS02 amplicons were subsequently digested using enzyme MSP I (New England BioLabs) and Bovine Serum Albumin (BSA)(New England BioLabs) at 37°C for one hour. Digested CPS02 amplicons were electrophoresed through a 1.5% agarose gel stained with ethidium bromide at 75 volts for 45 minutes, and then visualized using Luminary FX (FOTODYNE Incorporated). Positive and negative controls were run with each reaction. Trees containing true *J. cinerea* chloroplast DNA display a single amplicon band of 332 bp. In comparison, trees containing *J. ailantifolia* chloroplast DNA have a cleaved amplicon of 235bp and 97bp in length (Figure 2.1).

Samples that contained *J. ailantifolia* DNA and samples that could not be resolved in this hybrid test were removed from further analyses. Of the total 237 samples collected, thirty-one trees (13%) were verified to contain *J. ailantifolia* DNA.

3.2.6 Data analysis

Individuals that were missing more than 15% of data metrics were removed from the sample set. Variables were tested for statistical normality by evaluating skewness (asymmetry) and kurtosis (peakiness) using Pc-Ord v6.08 (MjM Software, Gleneden Beach, Oregon, U.S.A.). Where a skewness and kurtosis of zero represent a normal curve, remediation should be considered when skewness >1.
In order to evaluate the distribution of health, ecological, and growth parameters subpopulations were tested for between and among diversity of DBH, crown class, vigor, canker number (0.3 – 2.7 m) percent canker girdling, and number of epicormics using an ANOVA for the continuous data and contingency tests for ordinal data, performed in JMP, v.10 (SAS Institute Inc., Cary, NC). The ANOVA was used to test variation between continuous data and contingency tables and Fisher exact tests were used for the comparison of nominal and ordinal data. To evaluate the likelihood of persistence of the butternut species, degree of recruitment was graphed into a population pyramid, and mean DBH was regressed with a rating of overall vigor using a logistic regression.

To test the hypothesis that butternuts with deep-fissured bark are more resistant to *Oc-j*, a student t-test compared bark type with vigor, number of epicormics, number of trunk cankers, and percent girdled. A multivariate discriminant analysis separated bark type and discriminated among canker number, epicormic number, DBH, and healed cankers. To test the null hypothesis that upland and riparian trees vary in disease severity an ANOVA contrasted vigor, number of epicormics, number of trunk cankers, and percent girdled to trees grouped as upland or riparian. A multivariate discriminant analysis performed in JMP v.10 separated upland from riparian trees and discriminated between total epicormic, DBH, and number of cankers.

As a product of unreliable introduction dates for butternut canker fungus *Oc-j*, tree samples of DBH cohort 25.6 cm (26 yrs) through 37.7cm (40 yrs) were removed from the dataset to allow for a stronger comparative analysis to test whether trees growing after *Oc-j* introduction are equally as fit as tree established pre *Oc-j* introduction. The DBH range removed was calculated using Frelich’s model to calculate the age of
butternut trees from DBH. Trees five years older than the first report of butternut canker in the Northeast were separated from trees five years younger than the first report of butternut canker in the northeast. Similar to sessile habitat, butternut trees pre and post *Oe-j* introduction were compared using an ANOVA and contingency tests to delineate vigor, canker count, and habitat. These calculations were performed in JMP 10.

Hybrid butternut trees are reported to be more resistant to *Oe-j* compared to true butternut trees. This hypothesis was tested by ANOVA using virtues of vigor (number of epicormic, crown class, canker number, DBH, crown dieback, and habitat) to compare between both hybrid and true butternut trees. A discriminant analysis categorized butternut from hybrid trees and ordinated epicormic count, DBH, and canker count.

3.3 Results

3.3.1 Sub-population summary

In total 252 trees were sampled from 19 sites across NY, VT, NH, ME (Table 3.1). When sample unit outliers were detected by skewness and kurtosis only epicormic count had a skewness > 1. An epicormic outlier datum was removed from the data set, which remedied the overall skewness for epicormic count. Hybrids were identified (see 3.3.3), removed from the dataset and subpopulations were tested for variation using regressions and contingency tests. ANOVAs tested the significance of each regression and Fisher's exact test interpreted the significance for contingency tables. Diameter at breast height (DBH) and the number of epicormics per tree differed significantly between sub-populations following a Bonferroni correction (*P*<0.0001). The total number of cankers was not significantly different between subpopulations following a Bonferroni
correction ($P=0.0002$). Contingency tests revealed significant variation between subpopulations for crown class ($P<0.0001$) and vigor rating ($P<0.0001$) but not for percent girdled ($P=0.0034$). The Bonferroni correction for the sub-population significance tests were based on 271 pairwise comparisons at an adjusted $P$-value of 0.00018. The rating of tree vigor had no significant relation with the number of cankers found on a tree (Figure 3.1)

### 3.3.2 Butternut dendrochronology

Eight core samples were obtained with corresponding DBH measurements. The model applied to published DBH and age performed poorly. Two locations, Butternut Valley and Clinch had respective DBH's of 29.5cm and 29.3cm and average ages of 43 and 32 years old (Clark et al., 2008). Using DBH, Frelich's model predicted the ages of Butternut Valley at 30.2 and Clinch at 29.9 years old. No further tests were pursued due to a lack of model applicability.

### 3.3.3 Butternut compared to hybrid trees

In selecting sampling sites, reports of butternuts located in or near towns were generally avoided. Established towns, and even long established farm stands are prone to having nursery trees planted nearby, increasing the likelihood of *J. ailantifolia* or butternut hybrids nearby. Because we are monitoring the genetic diversity of *J. cinerea*, we wanted to avoid including Japanese walnut or hybrids in this study. Proximity to human modified landscapes can be a predictor of butternut hybrids (Hoban et al., 2012). One site visited clearly contained hybrid butternut trees. Sites containing putative hybrid
trees were avoided. Of the total 252 trees sampled, 31 were discovered by molecular methods to contain *J. ailantifolia* chloroplast genes. Two sampling locations contained only hybrid trees totaling 24, the remaining seven hybrids were detected growing among naturalized butternut stands (Table 3.1). A comparison of butternut and hybrid trees revealed a significant difference in DBH where hybrid trees had a greater diameter ($P<0.0001$; Table 3.2). Hybrid trees were found to have significantly deeper fissured bark ($P=0.0076$; Figure 3.2), a higher likelihood of growing in upland habitats ($P=0.0029$; Table 3.1), increased vigor rating ($p<0.0001$; Figure 3.3), fewer cankers ($P=0.008$), and less crown dieback ($P=0.0003$) when compared to true butternuts (Table 3.2). Hybrid trees did not vary from true butternuts in the number of epicormics ($P=0.2772$) or crown class ($P=0.2527$). A discriminant analysis of hybrid and true butternut trees confirmed univariate results in that hybrid trees were positively ordinated hybrid trees, total number of cankers were positively ordinated with butternut trees, and neither hybrid nor butternut were influenced by epicormic number (Figure 3.4).

### 3.3.4 Recruitment

Analyses on the magnitude of butternut recruitment revealed overall mean DBH of 35.2 cm. Average DBH also represented proportionally the largest age (Figure 3.5). A box plot for the logistic regression of DBH and vigor was non-significant ($P=0.1605$; Figure 3.6), but indicated a trend of positive association between DBH and vigor. DBH and total canker number were found to be negatively associated where trees of smaller DBH having significantly more cankers per tree ($P=0.0053$; Figure 3.7).
3.3.5 Bark Phenotype

Vigor rating and bark phenotype are non-related as evaluated by contingency test ($r^2=0.02$) and fisher's two-sided exact test ($P=0.1739$). Total epicormic count and bark phenotype are also not related ($P=0.3657$; Table 3.2) and canker number was significant to bark phenotype following a Bonferroni correction ($P=0.0345$; Figure 3.8). The percentage of girdling due to canker infection was significantly related to bark phenotype ($P=0.0041$; Figure 3.9) with highest level of girdling occurring in shallow fissured bark. A canonical plot discriminant analysis of bark type revealed DBH and epicormic count as component positively associated with deep-fissured bark type (Figure 3.10). The CDA had F-ratios of 0.6685 and 0.9986.

3.3.6 Sessile habitat

Upland and riparian habitats vary considerably in resource distribution. No significant difference was found in the vigor between trees growing in upland versus trees growing in riparian zones ($P=0.2058$), nor did habitat type significantly differ for the total number of cankers ($P=0.1521$) or the percent girdled ($P=0.1176$). There was, however, a difference between upland and riparian sites for the number of epicormics per tree. Riparian trees had significantly more epicormics when compared to upland trees ($P=0.0005$; Table 3.2). A discriminant analysis separating habitats found that epicormics and canker number positively correlating with riparian trees, whereas there was a positive correlation between DBH and upland trees (Figure 3.11). A heat map overlaying habitat on a regression of DBH and canker number reveals a trend where trees growing in upland habitats tended to be larger with fewer cankers. Contrary to this, trees growing in riparian
habitats had a tendency to be smaller with more cankers (Figure 3.12)

3.3.7 Post and pre Oc-j introduction

The separation of butternut trees that had established prior to Oc-j introduction and post Oc-j introduction resulted in the removal of 63 trees from our dataset representing the DBH cohort of 25.6 to 37.7cm. Trees smaller than 25.6 cm DBH had significantly more cankers when compared to trees larger than 37.7 cm DBH ($P=0.0069$; Table 3.2). Furthermore there was a significant effect of habitat on the presence of trees in the younger or older cohorts. Trees from the younger cohort (<25.6cm) were more frequently observed in riparian habitats than the older cohort (>37.7) of which were more frequently observed in upland habitats ($P=0.0001$).

3.4 Discussion

3.4.1 Sub-population summary

The variables recorded from each tree can be grouped and typified by a normalized curve. Split into sample locations, measurements of vigor, DBH, and crown class varied significantly. This variation between subpopulations allowed for hypothesis testing between subpopulations, while the dataset as a whole remained normal. The number of cankers per tree and the percent girdled did not vary between sample locations indicating an even spread of butternut canker across the northeast. This corroborates with the nearly 100% canker incidence rate observed by Bergdhal and Bergdhal (2010). Despite the uniform presence of butternut canker, Bergdhal and Bergdhal note a variation in disease severity. This corresponds nicely with the significant variation in vigor and
crown class observed in the present study. Speculation of resistant butternut trees is not a novel concept (McKenna et al., 2011; Orchard et al., 1982; Ostry and Woeste, 2004; Ostry and Moore, 2008), although the mechanism of resistance remains uncertain. The present study was not designed to test the mechanism of resistance, however, our approach allowed for empirical testing of disease pressure and vigor between sampling locations, demonstrating that butternut canker is uniformly present across the Northeast, although ratings of vigor vary among subpopulations.

3.4.2 Butternut dendrochronology

The ability to predict butternut age using the black walnut DBH-age model is questionable. Frelich’s model performed poorly when estimating age using average DBH from two sample sites (Butternut valley and Clinch) published by Clark et al. (2008). DBH should be used only as a very broad estimator of tree age, perhaps 30 year cohorts (S. Clark, personal communication). A model specific to butternut should be developed for more appropriate estimations of tree age. Such a model would allow researchers to ask questions specifically related to tree age, rather than DBH.

3.4.3 Butternut and hybrid trees

Despite an attempt to avoid the sampling of hybrid trees, 31 trees were confirmed to contain *J. ailantifolia* chloroplast DNA. Due to the attempt of sampling pure butternut exclusively, one should heed caution when comparing the ecological and phenotypic differences to hybrid trees. In addition to this only a single, maternally inherited chloroplast gene was used to identify hybrids. This molecular approach was able to
identify hybrids with a paternal *J. cinerea* and maternal *J. ailantifolia*. This approach is likely to be sufficient for our analytical needs as most hybrids contain maternally inherited *J. ailantifolia* chloroplast (Hoban et al., 2009). Complex backcrosses and hybrids not detected by our assay would likely have a minimal influence on the large sample size used in the present study.

Butternut bark is light-grey with flat, closely furrowed ridges (Figure 3.13). Exceptions to this phenotype have been noted and while the present study did not explicitly test for phenotypic differences between hybrids and true butternut, it should be noted that hybrid trees are more likely to have a darker-grey and deeply fissured bark (Fig. 3.2). This is likely attributed to the larger average DBH observed in hybrid trees. *J. ailantifolia* was imported as a food crop and ornamental. For this reason it is far more likely to encounter a hybrid tree in an upland habitat rather than within a riparian zone. Hybrid trees were observed to have a higher average vigor, less crown dieback, and fewer cankers. The increased resistance displayed by hybrid trees supports the hypothesis that *Oc-j* is an exotic fungus (Furnier, 1999). It appears that hybrid trees are similar to butternut trees in their requirement for light. Butternut trees and hybrids are indistinguishable by the number of epicormics grown, or the relative crown class.

### 3.4.4 Recruitment

*Oc-j* affects butternut trees of all ages, of which the youngest are most susceptible. This hypothesis was not supported when regressing vigor rating to DBH (Figure 3.14). An alternative, possibly less subjective approach to test whether younger trees was more impacted by *Oc-j* differed in conclusion. Younger trees were found to
have more cankers per tree than did older trees (Figure 3.7) and proportionally fewer small trees were sampled. One explanation for the absentee young cohort can be somewhat explained by the sampling method; older butternut trees are easier to locate and identify than younger trees. Alternate possibilities are that Oc-j kills young butternut trees faster than older trees as susceptible allele combinations have not been purged from the genepool. A more appropriate method of testing this hypothesis would be the ex situ inoculation of a variety of DBH cohorts.

3.4.5 Bark Phenotype

A variety of bark phenotypes, from light grey with shallow fissures to darker grey with deeper fissuring has been observed. Initially suggested in 2003, dark grey deep-fissured trees appeared to be less affected by butternut canker than nearby light-grey shallow fissured types (Ostry et al., 2003). This hypothesis was further tested, and it was found that the dark phenotype had significantly fewer canker disease symptoms than the corresponding light phenotype (Ross-Davis et al., 2008). No association existed between fissure depth and canker symptoms. The present study used a combination of color and fissure depth to phenotype trees. Trees with a shallow phenotype were more likely (P=0.0041) to have extensive canker girdling than did trees with a deeper fissure phenotype (Figure 3.9). No other associations were found between bark phenotype and canker number, epicormic number, or vigor rating. Diameter at breast height was the best predictor of bark phenotype where trees of a larger DBH also had deeper fissuring. Future studies should use bark colour rather than fissure depth to phenotype trees.
3.4.6 Sessile habitat

Butternut trees were equally affected by butternut canker whether they were growing in an upland or riparian habitat, as they did not differ in canker number, vigor or percent girdled. Interestingly, the number of epicormics per tree was significantly greater in riparian habitats than in upland habitats. This can likely be attributed to the closed canopy generally observed in upland habitats. Butternut trees are shade intolerant and epicormics initiated under a closed canopy would likely not survive (Figure 3.15). Riparian zones generally have a less developed canopy allowing butternut epicormics enough light resource to persist. In light of differing genotypes, conservation policy should consider the preservation of both upland and riparian trees.

3.4.7 Pre and post Oc-j introduction

Butternut trees that were established prior to the first reports of Oc-j were not likely to be more vigorous than trees established after reports of Oc-j. The older cohort was however, more likely to have fewer cankers per tree than the younger cohort (Table 3.2). This combination of difference in canker number but no difference in vigor is puzzling and should emphasize that canker number alone is not an appropriate measurement for decisions of management.

3.4.8 Future butternut research

The current state of uniformity among butternut trees of the Northeast allow for a range-wide approach to management. Butternut is being ebbed from mature forest stands due to their relatively short life-span and intolerance to shade. Natural disturbances like
flood and fire will aid in the recruitment of new butternut stands. Breeding for natural resistance seems futile based on the magnitude of disease presence and lack of identified resistance. A more effective approach for resistant trees is to hybridize butternut with less susceptible *Juglans* species. Hybrid trees often phenotypically resemble true butternut and should be actively introgressed with naturalized populations. Rapid introgression will reduce the total loss of genetic variation. Future research into niche modeling could provide insight on whether hybrid can fulfill a similar ecological role as butternut has in the past.
Figure 3.1. Non-significant relationship between categorical rating of tree vigor with the number of trunk cankers between 0.3 and 2.7 meters ($P=0.0686$)
Figure 3.2. Contingency test comparing butternut and hybrid bark phenotype. Distribution along X-axis displays sampling disproportion. Hybrid trees tended to have significantly deeper fissured bark compared to true butternut ($P=0.0076$)
Figure 3.3. Contingency table of vigor rating for butternut and butternut hybrids ($P<0.0001$)
Figure 3.4. Discriminant canonical analyses of butternut and hybrid trees. Ellipses each contain 50% data points for butternut and hybrid trees.
Figure 3.5. Distribution of northeastern butternut trees based on diameter at breast height. Red line indicates the first reports of butternut canker in the northeast with respect to DBH cohort.
Figure 3.6. Vigor rating for butternut with respect to diameter at breast height
Figure 3.7. Linear regression of diameter at breast height and the number of trunk cankers. Line of best fit indicated ($r^2=0.04$) surrounded by the standard error shaded in blue. Tree size is inversely correlated with canker number where smaller trees have more cankers than larger trees ($P<0.0053$)
Figure 3.8. Mean number of cankers for shallow, intermediate and deep bark phenotypes ($P=0.0345$)
Figure 3.9. Ordinal rating of butternut canker girdling organized by bark phenotype. Distribution across X-axis represents proportion of each bark phenotype. Shallow bark phenotype have significantly more canker girdling than do deep or intermediate phenotypes ($P=0.0041$)
Figure 3.10. Discriminant canonical plots separated by bark phenotype. Normal ellipse region contain 50% of each bark phenotype with an error of 37% misclassified data.
Figure 3.11. Discriminant canonical plots separated by habitat type. Normal ellipse region contain 50% of each habitat type with an error of 36% misclassified data.
Figure 3.12. Distribution of habitat on a regression of diameter at breast height and canker number
Figure 3.13. Comparing the deep-fissured (a) and shallow fissured (b) bark phenotypes in butternut
Figure 3.14. Cumulative probability plot for diameter at breast height regressed with vigor rating
Figure 3.15. Photographs contrasting butternuts growing in mature upland forests (a) and open riparian flood plains (b)
### TABLE 3.1. SAMPLING LOCATIONS AND GROWTH CHARACTERISTICS

<table>
<thead>
<tr>
<th>Site</th>
<th>Lat.</th>
<th>Long.</th>
<th>N (%)</th>
<th>Bark</th>
<th>DBH (^{2U})</th>
<th>Habitat (^{1})</th>
<th>Canker (^{2U})</th>
<th>Epicormic (^{2U})</th>
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</thead>
<tbody>
<tr>
<td>ABC</td>
<td>44.655</td>
<td>-71.566</td>
<td>20</td>
<td>S</td>
<td>27.2 (_{CD})</td>
<td>R</td>
<td>4.8 (_{BC})</td>
<td>0.5 (_{C})</td>
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<td>BB</td>
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<tr>
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<td>0.5 (_{C})</td>
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<td>D</td>
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<td>0 (_{ABC})</td>
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<td>6.2 (_{A})</td>
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<td>CHA</td>
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<td>D</td>
<td>40.4 (_{U})</td>
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<td>-71.622</td>
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Latitude and longitude measured in degrees North and West
U - Levels not connected by same letter are significantly different.
1 - Mode
2 - Mean

81
### TABLE 3.2. ASSOCIATIONS BETWEEN GROWTH CHARACTERISTICS AND PHENOTYPE

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<th></th>
<th>N</th>
<th>DBH $^2$</th>
<th>Std. Dev.</th>
<th>Canker$^2$</th>
<th>Epicormics$^2$</th>
<th>Crown dieback$^1$</th>
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<td><strong>Oc-j introduction</strong></td>
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<td>Pre-Oc-j</td>
<td>82</td>
<td>52.9</td>
<td>15.7</td>
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<tr>
<td>Post-Oc-j</td>
<td>81</td>
<td>14.4</td>
<td>6.5</td>
<td>3.7</td>
<td>0.9</td>
<td>&lt;10%</td>
</tr>
<tr>
<td><strong>Hybrid Status</strong></td>
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<td>Butternut</td>
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<td>32.9</td>
<td>1.4</td>
<td>4.5</td>
<td>1.7</td>
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<tr>
<td>Hybrid</td>
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<td>4</td>
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<td>19.1</td>
<td>5.3</td>
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<td>&lt;10%</td>
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<td>Intermediate</td>
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<td>38.3</td>
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<td>Deep</td>
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<td>49.4</td>
<td>18.4</td>
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<td>2.1</td>
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<td>18.7</td>
<td>4.3</td>
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<td>10-50%</td>
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</table>

*Significance indicated in bold
1 - Mode
2 - Mean
CHAPTER 4: CONCLUSION

This study set out to characterize a community of butternut in the northeastern United States by addressing recruitment, health, hybridization and genetic diversity. Furthermore, data presented here aims to compliment studies of a similar nature by applying previously addressed questions to a new location and sampling scheme. Butternut, threatened by an exotic fungus, provides researchers with an opportunity to observe the effects of an epidemic and its relation with population fluctuation and persistence. More practically, this study provides a general health assessment and spatial genetic structuring analysis for application in management and conservation by addressing these two questions:

1. How physically and genetically healthy are communities of northeastern butternut?

2. Should conservation policy account for population structuring?

The health of a community can be measured in many ways. The International Union for Conservation of Nature (IUCN) assesses the conservation status of species and publishes a Red list of endangerment status. NatureServe, an IUCN Red List partner, evaluated butternut as Vulnerable (S3) in the states of New Hampshire and Vermont, and Apparently Secure (S4) in New York (NatureServe, 2013). Nationally butternut is listed as NatureServe's lowest priority: Apparently Secure (S4). Based on the results presented in this thesis, I argue that these listings provide a false impression and should be updated.

Health factors considered in the present study include magnitude of infection, recruitment, population size, genetic diversity, and a variety of vigor measurements. These summarizing factors are the product of past community perturbations which can, in
turn, be used to anticipate future community fluctuations. So, how healthy are northeastern butternut?

Mature trees are not recruiting sufficiently for butternut persistence. Genetic data provide an empirical approach to what otherwise may be a subjective community analysis. Multiple populations from New Hampshire and New York display the genetic signatures of inbreeding, a process associated with small effective populations. The consequences of inbreeding are seed germination failure, a decline in reproductive efficiency, and reduced offspring survival rates (Henry, 2006). Butternut demographics corroborate this evidence of low recruitment (Figure 3.5).

In addition to low recruitment, genetic homogeneity caused by inbreeding can increase an organism’s susceptibility to environmental variation and disease. For butternut, Oc-j affects trees of all ages. Younger trees have more cankers and likely succumb to butternut canker faster than well-established trees. Since the introduction of Oc-j, butternut has been under selective pressure for tolerance to butternut canker. Today, butternut stands comprise of mature trees that have tolerated Oc-j to varying degrees. The most susceptible trees likely succumbed to butternut canker rapidly, although insufficient time has passed since Oc-j introduction to purge susceptible alleles by genetic drift. While younger trees are more susceptible to butternut canker due to their lack of establishment, I argue that the most susceptible genotypes are still being recruited. This results in further reducing the already low recruitment of butternut seedling. It is difficult to say whether this selective pressure will leave sufficient genetic variation to prevent severe inbreeding depression and eventual population collapse.

The severity of a bottleneck can only be determined following the event. For
example Hoban et al (2010) found the genetic signatures of a bottleneck during Pleistocene-era range-shift more significant than contemporary decline. Considering Oc-j introduction dates, butternuts effective population size, and generation overlap, genetic drift has not had sufficient time to leave the genetic signatures of a bottleneck. We are on the cusp of a bottleneck as indicated by a lack of recruitment, inbreeding caused by small population size, and the regular presence of butternut canker.

Breeding for resistance depends on the end user imperative (Michler et al., 2006). More clearly, if we do not have a motive for saving butternut trees, should energy be invested in breeding resistant butternut? In general, literature often argues the usefulness of butternut wood for veneer, or the nut for its oily flavor, or traditional medicinal applications of various parts, and finally an appeal to tradition. I believe that a butternut hybrid may serve all these motives. Breeding hybrids for resistance was successful with American chestnut. Butternuts have an advantage in that F1 hybrids are similar to butternut in phenotype. The possibility of hybrid introgression should be welcomed on account that: 1) hybrid trees may be phenotypically indistinguishable from pure butternut (aside from their vigor); 2) hybrid trees can establish in both upland and riparian habitats; and 3) hybrid trees demonstrate more tolerance to Oc-j than do butternuts. Saving ex situ butternut germplasm for archival purposes is important for future reference but too much importance is placed on breeding resistance in pure butternut. A rapid introgression of hybrid trees into butternut stands will result in the preservation of a larger butternut genepool.

Managing disease in naturalized forests is difficult due to scale. Even more so, managing a rare species at large-scale has inherent difficulties. The sampling scheme
used in this study was effective at locating a large number of trees, but provides room for errors. Land-owners who responded to a call for butternut are likely to have responded only if a butternut stand was mature enough for identification. Younger stands may have been missed due to difficulties in identifying saplings. While genetics can circumnavigate some flaws associated with census data, microsatellite loci proved to be costly and laborious. Modern genetic techniques like genotype by sequencing (GBS) should be applied to allow for streamlined population genetics. The development of exome microarray chips could be effectively applied in hybrid and resistance breeding programs.

This study has used empirical findings to demonstrate that current NatureServe policy is outdated and should be revised to convey current developments in butternut communities. The genetic cohesiveness of butternut in the northeast allows policy and conservation to treat butternut of the northeast as panmitic. A lack of spatial geographic structuring permits the movement of putatively resistant clones across the northeast without risk of significantly disturbing butternut genetics. To the north of my sampling area, Canada has listed butternut as endangered and to the south, reports of widespread mortality well exceeding the threshold of ICUN's vulnerable species. Butternut populations in New York are not secure, and the NatureServe ranking should reflect rankings in surrounding states. Genetic cohesiveness and widespread disease on butternut of the northeast present a situation where careful monitoring and management, along with introgressive hybridization, can prevent population collapse and extinction.
REFERENCES:


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