GENETICALLY–MEDIATED LEAF CHEMISTRY IN INVASIVE AND NATIVE BLACK LOCUST (*ROBINIA PSEUDOACACIA* L.) ECOSYSTEMS

A Thesis
by
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Abstract

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Black locust (*Robinia pseudoacacia* L.) is one of the few examples of an intra-continentally invasive species. Native to the Southern Appalachians, black locust is currently naturalized on every continent except Antarctica. Few genetic studies have been conducted on black locust and none compare North American invasive and native populations. Chapter 1 is a summary of what is currently known about the taxonomy of the species and the genetic structure of black locust populations. Because black locust is a nitrogen-fixing tree, it has the potential to greatly alter the ecosystems in which it invades. The goal of Chapter 2 is to characterize the genetic and chemical variation among populations throughout the native Appalachian region and in two invaded regions in the Northeast and Midwest regions of the U.S. Understanding the role that genetic identity contributes to altering ecosystem function may help elucidate how invasion can cause changes across local and regional scales. To assist in understanding the impact of black locust across ecosystems, it is essential to

develop rapid and non-destructive means of estimating genetic and chemical characteristics. The focus of Chapter 3 is to test whether or not *in situ* leaf spectra-based models can be used to accurately determine leaf chemistries and predict genet membership. Understanding the chemical, genetic, and spectral differences among black locust genets is important for gaining a better understanding of the ecosystem impacts and invasive potential of this species.

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Foreword

Chapter 2 and Chapter 3 of this thesis will be submitted to *Biological Invasions*, an international peer-reviewed journal published by Springer International Publishing; they have been formatted according to the style guide for the journal.

CHAPTER 1: GENUS *ROBINIA* L. – TAXONOMY AND GENETIC DIVERSITY

The genus *Robinia* is characterized by a variety of woody legume species, both shrubs and trees, all with pinnately compound leaves and diadelphous flower morphologies (Isely and Peabody 1984). *Robinia* includes four species, *R. hispida*, *R. neomexicana*, *R. viscosa*, *R. pseudoacacia*, and their four presumed hybrids (Table 1.1). Additionally, within *R. hispida*, *R. neomexicana* and *R. viscosa*, multiple horticultural varieties are recognized. All of the *Robinia* species are native to temperate North America. The pink flowering species *R. hispida*, *R. neomexicana* and *R. viscosa* have weak interspecific morphological distinctions; however, they group into geographically distinct species (Isely and Peabody 1984). *R. pseudoacacia* is the only species to produce white flowers and is morphologically distinct from the other *Robinia* species (Vinogradova et al. 2013).

Robinia pseudoacacia, known as black locust or false acacia, is the best studied of the *Robinia* species due to its widespread distribution and invasive status. Black locust is an open-pollinated tree species pollinated predominately by insects in the order Hymenoptera. The physical separation of the stigmata and antheridia, as well as protogynous flowering, promotes out-crossing as the dominant mating system (Surles et al. 1990). However, due to variations in flower maturation, self-fertilization can occur through geitonogamous selfing. Evidence of inbreeding depression has been demonstrated in uniparental pollination experiments by Yuan et al. (2013), who also

argued that inbreeding depression mostly likely acts as a post-pollination barrier to self-fertilization. In black locust populations experiencing inbreeding depression, Yuan et al. (2013) found high rates of seed abortion and lowered plant fitness.

Polyploidy Status

Black locust has been found to be both 2n=20 and 2n=22 (reviewed by Cierjacks et al. 2013). Additionally, stable tetraploidy can be induced by colchicine treatment (Ewald et al. 2009). Unlike *R. viscosa* and *R. hispida*, triploidy has not been documented in black locust. However, both Surles et al. (1989) and Liesbach and Schneck (2004) found unusually high numbers of loci per enzyme with some enzymes appearing to be duplicated. Such loci patterns are consistent with patterns associated with polyploidy (Liesebach et al. 2004).

Genetic Diversity

Several studies have explored the genetic diversity and structure of black locust using allozyme (Surles 1989; Chang et al. 1998; Gu et al. 2010), isozyme (Liesebach et al. 2004) and chloroplast DNA (Liesebach and Schneck 2012) markers within the native and invasive ranges. In an early genetic study, Surles et al. (1989) collected seeds from 23 populations within the native range and quantified 40 loci across 18 different enzyme systems. Overall genetic diversity was high and was primarily due to high diversity within seed sources (88%). Higher degrees of genetic differentiation occurred at disjunct sites. Patterns of geographic differentiation could not be determined which Surles et al. (1989) attributed to widespread plantings from different seed sources. In a later study, Chang et al. (1998) used similar allozyme methods to determine the within-population genetic structure of black locust in the Coweeta Basin. Genetic patchiness

within populations would have been evident if black locust were out crossing through near-neighbor mating. Instead, Chang et al. (1998) found that black locust populations were dominated by asexual reproduction and that genet structure varied in size from 1200 m2 up to 10,000 m2.

Liesbach and Schneck (2004) compared genetic diversity between native North American populations of black locust and introduced populations from Germany, Hungary and Slovakia using isozyme markers. They found high within population genetic variation and low among population genetic variation from six Hungarian progeny. In contrast, German progeny had low within population genetic variation and high differentiation between populations. Liesbach and Schneck (2012) repeated the 2004 study using chloroplast DNA and additional native range populations and found 11 haplotypes that originated from two distinct lineages. The four populations from the native range had high genetic variation despite having a low average number of haplotypes per population. Genetic diversity was lower within the introduced populations than within the native populations. Significant differentiation was found among populations within the native range (Gst = 58.4 ± 16.8%) and German populations (Gst = 41.9 ± 15.5%). However, genetic differentiation among Hungarian populations was not significant (Gst = 3.2 ± 6.4%). Three of the four populations were from the native range – the Northern and Southern portions of the Appalachian region (VA, TN and GA). The fourth isolated population was located in Southern Illinois. No geographic patterns of genetic diversity were discernible. However, the Georgia population was completely composed of one haplotype unique to the native range. In Tennessee and Virginia, two different haplotypes were found in each population. The

population from Southern Illinois, which would be considered outside of the North American native range, appeared to be an admixture of the 5 different haplotypes from the other three native populations.

Gu et al. (2010) used an allozyme marker approach to determine the genetic diversity of 19 black locust populations in China. Isolation by distance was the only distinct geographical pattern that was evident among populations. There was low genetic differentiation among populations (Gst =0.038). Additionally, genetic distances among populations were small, ranging from 0.015 to 0.065. Gu et al. (2010) attempted to link temperature and precipitation to allozyme variance parameters using a simple correlative analysis but found no significant correlations between genetic variation and any of the climatic variables tested. More recent studies have proposed improved spatially explicit methods of detecting patterns between genetic and environmental variables that may not evident from correlative analyses (e.g. Lee and Mitchell-Olds 2011).

In summary, previous studies agree that the majority of black locust populations are characterized by high genetic diversity with most of the variation occurring within populations. Isolation by distance is the most probable explanation for genetic differentiation between populations; however, none of the studies identified clear geographic patterns of genetic diversity. Genetic differentiation between populations varied based on geographical location and type of marker analysis employed. For instance, German populations studied by Liesbach and Schneck (2004, 2012) were highly differentiated based on isozyme and cpDNA data. Native range populations, however, showed low differentiation based on allozyme and isozyme markers and high

genetic differentiation based on cpDNA. To date, microsatellite genetic markers have not been used to test for genetic structure within North American populations of black locust.

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TABLE

Table 1.1: Recognized *Robinia* species and varieties from USDA Plants (plants.usda.gov, 2014)

Classification	**Scientific Name**	**Common Name**
Species	*Robinia* × *ambigua*	(hybrid)
Species	*Robinia hispida* L.	bristly locust
Variety	*Robinia hispida* L. var. *fertilis*	bristly locust
Variety	*Robinia hispida* L. var. *hispida*	bristly locust
Variety	*Robinia hispida* L. var. *kelseyi*	Kelsey's locust
Variety	*Robinia hispida* L. var. *nana*	bristly locust
Variety	*Robinia hispida* L. var. *rosea*	bristly locust
Species	*Robinia* × *holdtii*	Holdt's Locust (hybrid)
Species	*Robinia* × *longiloba*	(hybrid)
Species	*Robinia* × *margarettiae*	(hybrid)
Species	*Robinia neomexicana*	New Mexico locust
Variety	*Robinia neomexicana* var. *neomexicana*	New Mexico locust
Variety	*Robinia neomexicana* var. *rusbyi*	Rusby's locust
Species	*Robinia pseudoacacia* L.	black locust
Species	*Robinia viscosa*	clammy locust
Variety	*Robinia viscosa* var. *hartwegii*	Hartweg's locust
Variety	*Robinia viscosa* var. *viscosa*	clammy locust

CHAPTER 2: GENETICALLY–MEDIATED LEAF CHEMISTRY IN INVASIVE AND NATIVE BLACK LOCUST (*ROBINIA PSEUDOACACIA* L.) ECOSYSTEMS

Abstract Black locust (*Robinia pseudoacacia* L.) is one of the few examples of an intra-continentally invasive tree. As such, black locust ecosystems provide a unique opportunity to study invasive species in the absence of major geographical barriers. Leaf chemistry traits of invasive species, particularly in nitrogen-fixing plants such as black locust, can have strong influences on ecosystem processes and nutrient cycling. The purpose of this study was to measure regional and genetic variation in foliar (carbon, nitrogen, total phenolics, and lignin) and belowground chemistries (soil NH_4^+ and NO_3^-) in the native Appalachian region and in two invaded regions in the Northeast and Midwest regions of the U.S. Using eight microsatellite markers, I identified genets with multiple ramets for the Native (N=20), Northeast (N=22), and Midwest regions (N=25). All three regions exhibited high genetic diversity and little between-region genetic differentiation (1-3%). There was evidence of genetically-driven variation in leaf chemistries (explains 9-30% variation), and soil NH_4^+ and NO_3^- (explains 40-43% variation). However, weak correlations between leaf and soil chemistries suggest there was not a strong relationship between the aboveground and belowground chemistries. Therefore, variation in leaf chemistry is likely to be genetically influenced rather than solely soil N-induced. The presence of black locust increased soil NO_3^- by 177% in the Northeast, 39% in the Midwest, and 144% in the Native region. Black locust invasion

uniquely alters regions it invades by increasing soil nitrogen and the degree of soil nitrogen increase varies by genotype.

Introduction

Biological invasions can have ecosystem-level effects when the invading species introduce a new biological process, such as nitrogen fixation, into a system. For example in Hawai'i, the invasive nitrogen-fixing tree *Myrica faya* alters ecosystems by quadrupling the amount of nitrogen entering the system and increasing nitrogen (N) availability (Vitousek and Walker 1989). Likewise, invasion by another N-fixing tree, *Falcataria moluccana* increases both soil nitrogen and phosphorous in Hawai'i, causing forest compositional changes and altering stream quality by increasing nitrate levels by 600% (Hughes and Denslow 2005; Wiegner et al. 2013). Generally, the fates of the fixed nitrogen (as NH_4^+) are either to be 1) taken up directly by the roots , or 2) transformed into NO_2^- and NO_3^-, the latter of which can likewise be taken up by plants. As anions, NO_2^- and NO_3^- can be leached from soils and enter into water systems (e.g. Goldstein et al. 2006; Wiegner et al. 2013). Increasing nitrogen availability in N-limited ecosystems can increase soil fertility and net primary production (Vitousek and Howarth 1991; LeBauer and Treseder 2008). Despite increases in NPP with increasing N inputs, N-fixing invasives create a competitive environment that favors the establishment of fast growing plants that are often nonnative species (Simberloff and Holle 1999; Von Holle et al. 2006). Competition for resources can threaten native plant communities, especially in communities without a native N-fixing species (Simberloff and Holle 1999).

Approximately 10% of all invasive species in North America have N-fixing capabilities (Ehrenfeld 2003). In terms of negative ecosystem impacts, black locust is one of the worst 100 woody plant invaders worldwide (Von Holle et al. 2006). The species is native to the southern and central Appalachians and the Ozarks, but has naturalized in 47 states in the contiguous U.S. and is considered invasive in Europe, Asia, and throughout the Middle East (Von Holle et al. 2006). Due to its extensive range and N-fixing capabilities, black locust represents an important invasive species that could have large ecosystem level impacts. For instance, after a disturbance such as logging, black locust can grow rapidly and restore N soil pools back to pre-disturbance levels in less than 4 years (Boring and Swank 1984).

Genetics play an important role in determining the invasive potential and success of a species. For instance, high genetic diversity is thought to increase invasiveness by allowing species greater flexibility in their response to novel environmental conditions (Sakai et al. 2001; Allendorf and Lundquist 2003). Additionally, many proposed mechanisms of invasion include links between genetics and plant chemistry and/or functional roles. For instance, two commonly invoked mechanisms of invasion, evolution of increased competitive ability and optimal defense theory, hypothesize that genetic variability is required to permit an adaptive response to a novel environment (McKey 1974; McKey 1979; Blossey and Notzold 1995). Several studies have documented high genetic diversity in black locust populations both in the native range and invasive regions outside of North America (Surles 1989; Chang et al. 1998; Liesebach et al. 2004; Gu et al. 2010; Liesebach and Schneck 2012). High genetic

diversity may be a key characteristic that gives black locust the adaptive potential to expand its range.

Invasive species can alter ecosystem function through variation in litter chemistry by affecting detrital pathways, plant growth, and rates of nutrient cycling (Vitousek and Walker 1989). Additionally, invasive species can experience changes in functional traits post-invasion (Mooney and Cleland 2001; Lee 2002; Pigliucci 2005). For instance, shifts in plant resource allocation from defense to growth, as a response to mechanisms such as enemy release, would likely result in a reduction of defensive compounds (Keane and Crawley 2002; Cappuccino and Carpenter 2005; but see Agrawal and Kotanen 2003). Moreover, recent evidence shows that aggressive invaders such as black locust are likely to benefit from enemy release (Mitchell and Power 2003; Carpenter and Cappuccino 2005; Cappuccino and Carpenter 2005). Lower concentrations of defensive compounds such as polyphenolics can lead to faster decomposition and nutrient cycling (Hättenschwiler and Vitousek 2000), thus creating an environment that favors fast growing invasive plants. By creating patches of high nitrogen availability, invasive black locust stands often facilitate higher understory nonnative richness and abundance than do paired native stands (Von Holle et al. 2006).

Leaf chemistry is important to ecosystem level processes and can vary widely by genotype (Madritch and Hunter 2002; Schweitzer et al. 2008). Invasive species often experience novel selection pressures within introduced ranges that can influence genetically-mediated leaf chemistries (Mooney and Cleland 2001; Hierro et al. 2005). For instance, Blair and Wolfe (2004) using a common garden design compared native and invasive populations of *Silene latifolia,* a North American invasive plant introduced

from Europe, and found that invasive populations evolved to allocate plant resources to growth rather than to defensive compounds. Ecosystem impacts of genetically-mediated litter chemistry have been demonstrated in a variety of both intact and invaded ecosystems (Driebe and Whitham 2000; Treseder and Vitousek 2001; Madritch and Hunter 2002; Cadotte et al. 2010). Additionally, differences among genotypes are likely to be at least as important as are the number of genotypes because phylogenetic diversity is an important driver of ecosystem functioning (Srivastava et al. 2012). Understanding the importance of genetically-mediated variation in black locust chemistry is necessary to understand ecosystem effects of this invasive species.

This study is among the first to directly compare genetic and chemical characteristics of black locust between the native and North American invasive ranges *in situ*. The goals of this study were to 1) analyze the degree of genetic differentiation and genetic structure among and within regions, 2) determine whether or not variation in leaf and soil chemistries is genetically driven, and 3) broadly characterize important chemistries that can potentially alter nutrient cycling dynamics. Some degree of genetic differentiation and structure among regions may be apparent as a result of differences among geographic and environmental factors, as well as genetic isolation (Lee and Mitchell-Olds 2011). Significant inter-genet variations in leaf chemistry likely exist if leaf chemistry is indeed genetically-mediated. Additionally, concentrations of leaf chemistries indicative of poor leaf quality such as phenolics and C:N ratios are expected to be higher in the Native region than in the Midwest or Northeast regions. Although field observational studies are limited in their ability to untangle genotypic and environmental effects, understanding the *in situ* relationships between the effects is

essential for deriving ecologically relevant results from controlled experimental studies (Le Roux et al. 2013).

Methods and Materials

Field Site Description

In order to minimize environmental and climatic differences within sampling regions, I selected locations that fell within the Environmental Protection Agency's (EPA) level II regions (EPA 2010). The three regions sampled were 1) the Mixed Wood Plains region located throughout southeastern Minnesota, southwestern Wisconsin, 2) northern portions of the Atlantic Highlands region located throughout New Hampshire, Vermont, and the southern tip of Maine, and 3) the Southern Appalachian located throughout the mountainous areas of northwestern North Carolina, western Virginia, and eastern West Virginia (Fig 1), hereafter referred to as the Midwest, Northeast, and Native regions, respectively.

Leaf and Soil sample collection

I collected leaf and soil samples in the summer of 2013 from the three regions. Within each region, leaf tissue samples were collected from four individuals at 30 sampling locations. Sampling sites were separated by a minimum of 2 km to avoid oversampling ramets within the same genet (Chang et al. 1998; Mishima et al. 2009). All trees had a DBH (diameter at breast height) greater than 5 centimeters (cm). Leaves were stored in silica desiccant at 4°C before freeze-drying. After lyophilization, the leaves were stored at -20°C for further analysis.

From beneath the four trees sampled at each site, I collected a composite soil sample by pooling three 15 cm long soil cores taken one meter from the base of the tree.

Four control 15 cm soil cores were taken from an adjacent paired site that did not contain black locust. Paired control sites were located a minimum of 100 m from the nearest black locust tree to minimize influence of black locust litter and roots. Soils were sieved at 2 mm and then freeze-dried before analysis. All dried leaf and soil samples were ground using a ball mill.

DNA extraction and Microsatellite analyses

Genomic DNA was extracted from the leaves of each individual tree using the DNeasy Plant Mini kit reagents and protocol (Qiagen, Venlo, Netherlands). For genotyping analysis, eight pairs of co-dominate, species-specific, microsatellite markers were used (Lian and Hogetsu 2002; Mishima et al. 2009). Forward primers were labeled according to the M13-tailed PCR method (Schuelke 2000). PCR was performed with a final volume of 15 μL containing 7 μL of 2X Master Mix (Promega Corp, Madison WI), 0.3 μM M13-tailed forward primer, 0.3 μM M13 fluorescent dye (FAM, VIC, NED, PET), 0.6 μM reverse primer and 5-10 ng of DNA template. Reactions were performed using the touchdown PCR protocol described by Mishima et al. (2009) using Mastercycler Nexus (Eppendorf, Hamburg, Germany). Reactions were multiplexed with the GeneScan Liz 600 size standard and HiDi formamide (Applied Biosystems, Foster City, CA). Samples were then sent to the Georgia Genomics Facility (Athens, GA) where they were separated using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA). Each plate contained four quality control samples to allow for sequencing and scoring error detection and correction. Resulting chromatograms were scored using Peak Scanner software version 2.0 (Life Technologies, Carlsbad, CA).

Leaf and Soil Chemical Analyses

I analyzed leaf samples for total carbon, total nitrogen, lignin, and phenolics. Carbon and nitrogen were analyzed with combustion analysis using the Flash EA1112 elemental analyzer (Thermo Fisher Scientific, Waltham, MA). Total phenolics were measured as gallic acid equivalents (GAE) using the Folin-Ciocalteau reagent colorimetric assay with gallic acid standards (Cicco and Lattanzio 2011). Lignin concentrations were determined using a modified thioglycolic acid protocol (Suzuki et al. 2009). Kraft lignin (Sigma-Aldrich, St. Louis, MO) and purified barley were used for the standards and controls, respectively.

Soil ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 2M KCl. I measured ammonium using the indophenol blue method with ammonium chloride standard solutions (Mulvaney 1996). Nitrate was reduced to nitrite using vanadium (III) reduction and analyzed colorimetrically with Griess' Reagent (Doane and Horwáth 2003).

Comparison of genetic diversity and differentiation between native and introduced regions

The original 369 tree samples yielded 142 unique genotypes for statistical analyses. Individuals that failed to amplify at a minimum of 7 out of 8 loci were removed prior to all analyses (<2%). Descriptive regional genetic statistics were calculated using GenAlEx 6.5 (Peakall and Smouse 2012). Within-region statistics included the average number of alleles per locus (allelic diversity), average number of effective alleles per locus (effective allelic diversity), average number of rare alleles per locus, expected heterozygosity, observed heterozygosity, and the fixation index. Hardy-

Weinberg equilibrium was tested using a Bonferroni adjustment for each combination of loci and location. HWE calculations were performed in the R package PopGenReport (Adamack and Gruber 2014).

Genetic differentiation between regions was calculated using both Wright's F-statistic (Fst) and Goodman's Estimator (Rst). Both statistics of differentiation were included because, while Wright's F statistic is widely used, Rst assumes a step-wise mutational model that has been shown to be useful for co-dominant microsatellite markers (Wright 1943; Slatkin 1995). Analyses of Molecular Variance (AMOVA) were performed to partition variance into, among, and within inter-regional components, as well as estimate Fst and Rst.

Genetic structuring

A Principle Coordinates Analysis (PCoA) implemented in PopGenReport was first used to explore the range-wide structure. STRUCTURE (v.2.3) was used to identify the genetic structure of black locust across regions. STUCTURE is based on an admixture model that delineates clusters (K) of similar individuals by correlated allele frequencies from multi-loci genotypes (Pritchard et al. 2000). For each run, a burn-in of 20,000 followed by 50,000 Markov Chain Monte Carlo iterations was used. I tested K values from 1 to 15 with 20 runs per K value. Following the methods outlined by Evanno et al. (2005) the post hoc statistic ΔK was used to identify the most likely number of clusters. All ΔK values were calculated using the program Structure Harvester (Earl and vonHoldt 2011). STUCTURE outputs were corrected for issues of label-switching and multimodality using the program CLUMPP (Jakobsson and Rosenberg 2007).

Above- and belowground chemistry statistics

As a manipulative large-scale study within the continental native and invasive range was impractical, I used established black locust stands. Using established stands confounds black locust sites with spatial variation and site history prior to black locust establishment. To correct for these confounding factors, my collection sites were paired with sites without black locust. Such a paired sampling scheme has been shown to be useful for both ecosystem (Madritch et al. 2009) and community (Von Holle et al. 2006) responses to overstory plants. In order to insure dominance of genet litter inputs into belowground systems, I analyzed data from ramets with three or four genets and excluded those with only one or two genets. To isolate the effects of black locust on belowground soil chemistries, I subtracted the average chemistry of four paired control soil samples from the individual soil chemistries sampled below each genet (here forth referred to as ΔNO_3 and ΔNH_4).

To account for the hierarchical data structure and non-independence of genet replicates, I used linear mixed models to explain the influence of native status, region and genet identity on individual soil and leaf chemistries. I initially included measurements of diameter at breast height (DBH) for each tree in the model as a proxy for tree age, but variation in DBH did not explain any of the variation in leaf or soil chemistries and was therefore removed. Data were checked for normality and homoscedasticity. I ran all models using the lmer function in the R package lme4 (Bates et al. 2014). I used additional R packages to generate Type III ANOVA tables (car), and calculate AICc (AICcmodavg; Fox and Weisberg 2011; Mazerolle 2013). For each chemistry (soil and leaf), I ran three models: 1) reduced model (no fixed effect) and

genet identity (random effect), 2) region (fixed effect) and genet identity (random effect), and 3) native status (fixed effect) and genet identity (random effect). Based on the methods described by Nakagawa and Schielzeth (2013), I used the R package MuMI to calculate the marginal and conditional R^2. Briefly, R^2 marginal and R^2 conditional measure the amount of variation explained by the fixed effects and the full model, respectively. Pearson correlations were performed in R to analyze the relationships between soil and leaf chemistries and Wilcoxon rank sign test was used to compare paired and black locust soil samples.

Results

Genetic Analysis

From a total of 142 ramets, I scored an average of 141 for each of the eight microsatellite loci (Table 2.1). A total of 100 alleles were found across all regions. Out of 24 loci-site combinations, 5 deviated significantly from Hardy-Weinberg Equilibrium (HWE). There were no significant differences in frequency-based indices among the regions (Table 2.2). However, the Appalachian region had the highest allelic diversity and number of private alleles (alleles unique to the region; Table 2.2). For all regions, observed heterozygosity was lower than was expected based on HWE (Table 2.2). Positive fixation indices suggested that excess homozygosity was possibly the result of inbreeding.

Some genetic differentiation did occur among the three regions. The average differentiation across all regions based on Fst and Rst values were 1.8% and 3.2%, respectively. Regional pairwise Fst distances indicated that there was a 1.4% differentiation between the two invasive regions (p=0.002), 1.0% differentiation

between the Native and Midwest region (p=0.005), and 2.9% differentiation between the Native and the Northeast regions (p=0.001). STRUCTURE cluster analysis identified K=2 as the optimal number of clusters, however, there was no discernible geographical structure of the individuals (data not shown).

Leaf and soil chemistries

The majority of the variation in leaf chemistry was explained by regional effects. However, significant variation between genets explained 9-30% of the variation in leaf chemistry (Table 2.3, Fig. 2.2). Additionally, average chemistries were different among regions (Fig. 2.3). In terms of defensive compound concentrations, the Native region had the poorest leaf quality with the lowest amounts of leaf nitrogen and highest amounts of carbon, GAE, and lignin. The Northeast had similarly high amounts of GAE and lignin as did the Native region, however, the Northeast had significantly higher leaf nitrogen, lower carbon and lower C:N than did the Native range. Compared to the Native and the Northeast regions, the Midwest region had the highest leaf quality with the highest nitrogen and lowest carbon, GAE and lignin concentrations.

The analysis of the raw soil data taken from beneath individual trees demonstrated that although region had a significant influence on soil chemistry, genet identity explained a greater proportion of the variation than did region (Table 2.4). After soil extracts were normalized by control sites (ΔNH_4 and ΔNO_3), the majority of the variation in soil nitrogen was explained by genet and a small amount of variation was attributed to the native status of the tree (i.e. invasive or native). The presence of black locust was correlated with an increase in the average soil NO_3^- for each region compared to the average control NO_3^- (Table 2.5). The presence of black locust

increases soil NO_3^- by 177% in the Northeast, 144% in the Native region, and 39% in the Midwest. Soil NH_4^+ did not increase beneath black locust, and in fact, decreased by 14% in the Midwest region (Table 2.5).

Despite significant genet effects explaining variations in both leaf and soil chemistries, the above- and belowground chemistries were not strongly correlated with each other, suggesting the processes were not closely linked (Table 2.6). The effect of variation in leaf chemistry on belowground chemistry was most likely obscured, in part, by variation in belowground nitrogen fixation in the roots.

Discussion

The genetic analysis of black locust populations using microsatellite markers yielded comparable results to studies performed using allozyme markers (Surles 1989; Liesebach et al. 2004; Gu et al. 2010). Low genetic differentiation and high genetic diversity indicates there is gene flow among the regions, most likely as the result of multiple re-introductions. High gene flow is further supported by the lack of spatial genetic structure between or within regions. Interestingly, despite high observed heterozygosity (Ho) across all regions, heterozygosity is lower than what was expected (He) based on allele frequencies, which suggests low levels of inbreeding are maintained within regions. Yuan et al. (2013) found evidence of inbreeding depression in black locust. They argued that the high seed abortion and lowered fitness caused by inbreeding depression may act as a barrier to self-fertilization. Barriers to inbreeding and inbreeding depression are mechanisms through which black locust populations could maintain both high genetic diversity and low levels of inbreeding.

My data suggest that much of the variation in leaf chemistry is explained by genet identity. Differences in leaf quality (C:N, lignin, and GAE) follow trends predicted by mechanistic invasion hypotheses involving resource allocation. The native range had trees with higher concentrations of defense-associated compounds than did the invaded regions. Differences in herbivore pressure may be one possible explanation for the differences found in leaf chemistry among the regions. In the native range, black locust is the larval host of the locust leaf minor (*Odontota dorsalis* Thunburg), a native insect pest. While *O. dorsalis* infestation rarely results in tree mortality, defoliation of the trees over summer months reduces growth by half (Zheng et al. 2003). Resistance to *O. dorsalis* infestation has been observed in some native populations of black locust (Zheng et al. 2003). Additionally, high leaf nitrogen content is directly correlated to increased feeding by *O. dorsalis* (Athey and Connor 1989). Decreased herbivory pressure may be one possible explanation for the patterns in leaf chemistry found among the regions.

Genet identity explained the majority of the variation found in soil ΔNH_4 and ΔNO_3. Based on the percent increase from the control soils, black locust increased soil NO_3^- the most in the Northeast (177%) and Native (144%) regions. Soil NO_3^- increased by a lesser degree (39%) in the Midwest region. Differences in rates of anthropogenic N deposition between the regions would not likely explain the patterns found here, as the Midwest and Native regions have similar rates at ~4-6 kg/ha per year and Northeast region with lower rates at ~1-2 kg/ha (National Atmospheric Deposition Program, http://nadp.sws.uiuc.edu/). The smaller increase of soil NO_3^- in the Midwest was potentially due to the higher control soil NO_3^- levels which were 88-263% higher than

for the other two regions. High levels of soil nitrate have been shown to inhibit nodule formation and thus, N-fixation in black locust (Boring and Swank 1984; Röhm and Werner 1991). Soil NH_4^+ did not vary significantly from the controls in the Northeast or the Native regions. However, in the Midwest region, soil NH_4^+ under black locust was lower than was NH_4^+ in control soils, potentially indicating differences in the rates of belowground processes such as nitrification.

Leaf and soil chemistries were weakly correlated suggesting that litter inputs from black locust did not have a large impact on belowground NO_3^- and NH_4^+ concentrations. However, leaf litter inputs may be influencing other soil processes, such as respiration or nutrient transformation rates, which were not measured by this study. Importantly, weak correlations between leaf and soil chemistries support the finding that variation in leaf chemistry was genetically-mediated and was not entirely driven by differences in soil chemistries. As further support, Athey and Connor (1989) found that soil fertilization treatments did not result in significant differences in leaf nitrogen in black locust most likely due to down regulation of N-fixing with higher N, therefore resulting in similar N in the trees.

My research demonstrates that black locust invasion uniquely alters the region in which it invades by increasing soil nitrogen and that the degree of soil nitrogen increase varies by genotype. Such alterations in ecosystem function are consistent with other studies of invasive nitrogen fixing trees. For instance, the invasive nitrogen-fixing *Falcutaria moluccana* and *Myrica faya,* both introduced to different ecosystems in Hawai'i, increase soil nitrogen availability and also lead to increased nutrient cycling, nutrient availability, and leaf quality (Vitousek and Walker 1989; Hughes and Denslow

2005). Likewise, the N-fixing *Acacia* species introduced to South Africa increases both belowground nitrogen availability and nitrogen mineralization rates (Yelenik et al. 2004). Many of these N-fixing trees, including black locust, facilitate the establishment of other nonnative species and threaten native species abundance (Simberloff and Holle 1999; Hughes and Denslow 2005; Von Holle et al. 2006). Nitrogen-fixing trees alter ecosystems by fundamentally changing the environmental context in which native species exist. Understanding the role that genetic identity contributes to such environmental changes could help elucidate how changes occur across local and regional scales. Experimental studies such as reciprocal transplant and common garden designs can further pinpoint specific genetic and biogeochemical processes that lead to alterations in ecosystem function.

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TABLES

Table 2.1 Number of individuals scored for each marker (N), total alleles per marker (A) and significant departure from Hardy-Weinberg Equilibrium each combination of allele and location.

Marker	N	A	Midwest	Northeast	Native
RP035	141	10			
RP106	142	5			
RP200	142	20			
ROPS08	140	8			
ROPS02	139	18		***	***
RP109	142	14		***	
RP150	142	16	***	***	
RP206	140	9			

*** A Bonferroni adjustment of $\alpha = (0.5/24) = 0.0021$ was used.

Table 2.2 Frequency-based diversity indices average number and standard error (se) of alleles per locus (A), number of effective alleles per locus (Ae), observed heterozygosity (Ho), expected heterozygosity (He), and fixation index.

Regions	A	Ae	Ho	He	F	Private Alleles
Native	10.13	5.09	0.69	0.75	0.08	11
se	(1.33)	(0.84)	(0.04)	(0.05)	(0.04)	
Midwest	9.88	4.98	0.71	0.77	0.07	4
se	(1.47)	(0.70)	(0.05)	(0.04)	(0.06)	
Northeast	9.25	4.94	0.68	0.77	0.11	8
se	(1.32)	(0.67)	(0.03)	(0.04)	(0.03)	
Mean	9.750	5.002	0.692	0.760	0.086	7.7
se	(0.762)	(0.762)	(0.024)	(0.023)	(0.025)	(0.974)

Table 2.3 ANOVA (Type III) results from linear mixed models with region as the fixed effect and genet as a random effect. R^2 marginal (R^2m) and R^2 conditional (R^2c) represent the amount of variation explained by the fixed effect and full model, respectively. The difference between marginal and conditional R^2 (ΔR^2), reflects the variance explained by the random effect—genet identity.

Chemistry	χ^2	d.f.	p	R^2 m	R^2c	ΔR^2
%N	71.27	2	<0.0001	0.38	0.66	0.28
%C	190.01	2	<0.0001	0.62	0.79	0.17
%Lignin	115.39	2	<0.0001	0.39	0.48	0.09
C:N	136.48	2	<0.0001	0.56	0.78	0.22
GAE (mg/g)	78.28	2	<0.0001	0.43	0.73	0.30

Table 2.4 ANOVA statistics and coefficients of determination for the best models determined by AICc. R^2 marginal (R^2m) and R^2 conditional (R^2c) represent the amount of variation explained by the fixed effect and full model, respectively. The difference between marginal and conditional R^2 (ΔR^2), reflects the variance explained by the random effect—genet identity.

Soil Chemistry	**Fixed Effect**	**χ^2**	**p**	**R^2 m**	**R^2c**	**ΔR^2**
ΔNH_4^+	Native Status	4.22	0.040	0.04	0.44	0.40
ΔNO_3	Native Status	3.08	0.080	0.03	0.46	0.43
NH_4^+	Region	60.21	<0.001	0.32	0.56	0.24
NO_3^-	Region	14.54	<0.001	0.13	0.60	0.47
Control NH_4^+	Region	24.69	<0.001	0.18	0.55	0.37
Control NO_3^-	Region	92.94	<0.001	0.20	0.59	0.39

Table 2.5 Wilcoxon rank sign test comparing regional averages of NO_3^- and NH_4^+ from paired sites.

	NO_3^- (%)	**Control NO_3^- (%)**	**W**	**p**
Midwest	1.522	1.093	5805	***
Northeast	0.824	0.298	5662	***
Native	1.405	0.576	3796	***
	NH_4^+ (%)	**Control NH_4^+ (%)**	**W**	**p**
Midwest	0.575	0.670	3670	*
Northeast	1.003	1.085	3263	n.s.
Native	1.022	0.953	2908	n.s.

Table 2.6 Correlations between leaf and soil chemistry for the a) Native, b) Midwest, and c) Northeast regions.

Chemistry	Δ NH_4^+	Δ NO_3^-	NH_4^+	NO_3^-	Control NH_4^+	Control NO_3^-
Native						
%N	0.029	-0.107	0.019	-0.155	0.018	-0.020
%C	-0.214	-0.389***	-0.070	-0.417***	0.171	0.113
% Lignin	0.136	-0.150	0.034	-0.179	-0.057	0.017
C:N	-0.085	0.014	-0.046	0.035	0.026	0.022
GAE (mg/g)	-0.104	0.211	0.041	0.323***	0.130	0.064
Midwest						
%N	-0.158	-0.082	-0.239*	-0.129	-0.043	-0.062
%C	-0.241*	-0.143	-0.009	-0.165	0.202*	-0.036
% Lignin	-0.279**	-0.112	-0.089	-0.05	0.17	0.075
C:N	0.098	0.065	0.237*	0.072	0.094	0.01
GAE (mg/g)	0.23*	-0.098	0.168	-0.183	-0.064	-0.105
Northeast						
%N	-0.053	0.084	-0.055	0.031	0.025	-0.071
%C	-0.266*	-0.056	-0.177	-0.082	0.096	-0.02
% Lignin	-0.074	0.049	0.033	-0.11	0.083	-0.182
C:N	-0.009	-0.119	0.022	-0.056	0.002	0.088
GAE (mg/g)	-0.116	-0.027	-0.143	-0.065	0.036	-0.035

Significance: $p<0.001$ (***), $p<0.01$(**), $p<0.05$(*)

FIGURE LEGEND

Figure 2.1 Location of sampling sites. Each blue circle represents a sampling location. There are 30 sampling locations for region for a total of 90 sampling locations.

Figure 2.2 Principle Coordinates Analysis of the three regions using *Cavalli-Sforza* chord distances.

Figure 2.3 Descending average leaf chemistry by genet. Individual bars represent the average of three or four genets. Bar color (denoted by legend) indicates the region of origin of the genet.

Figure 2.4 Bean distribution plots of leaf chemistries for Appalachian, Northeast and Midwest regions. The solid bars represent the average for the region and the dotted line is the grand mean. Letters indicate the results of a Tukey's post hoc multiple comparisons analysis.

Figure 2.1

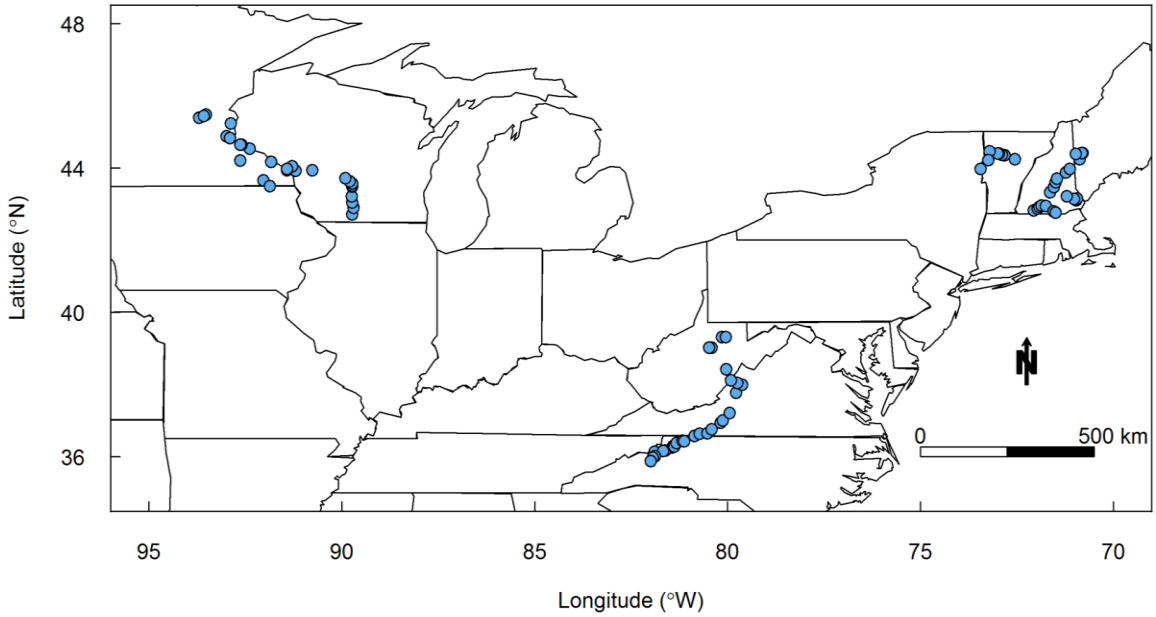


Figure 2.2

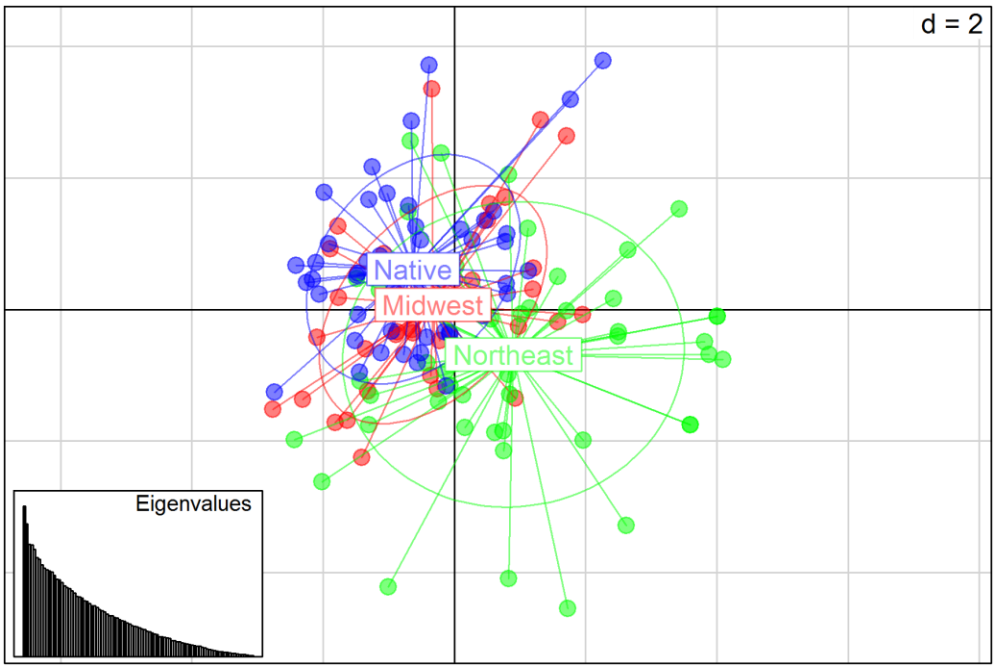


Figure 2.3

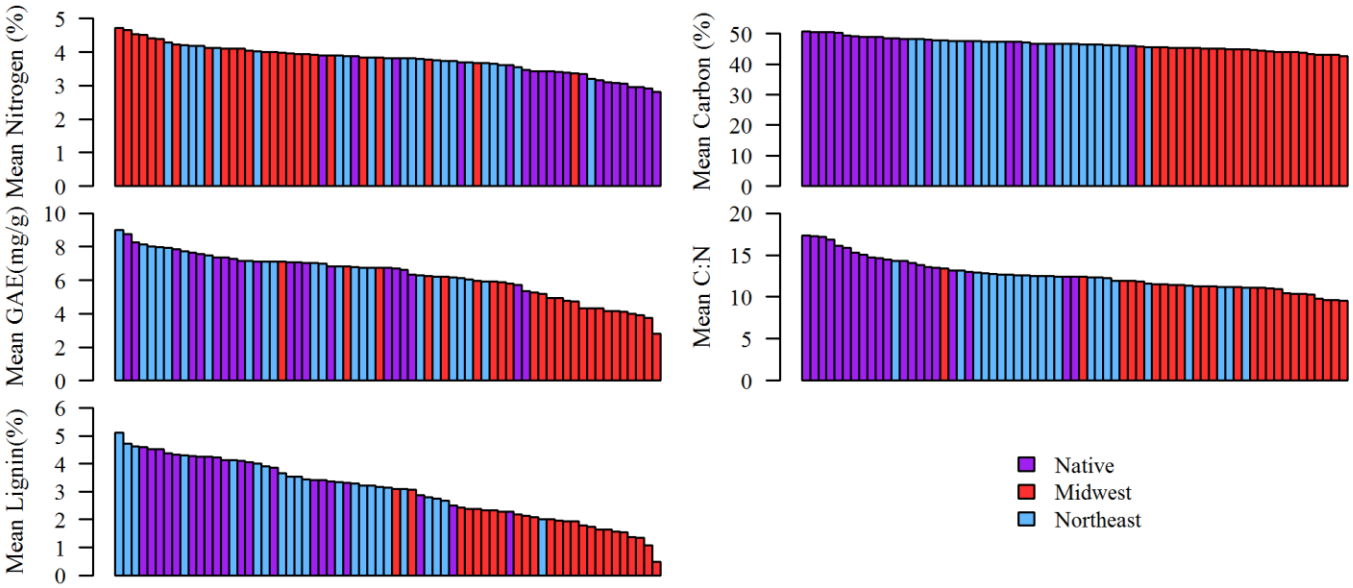
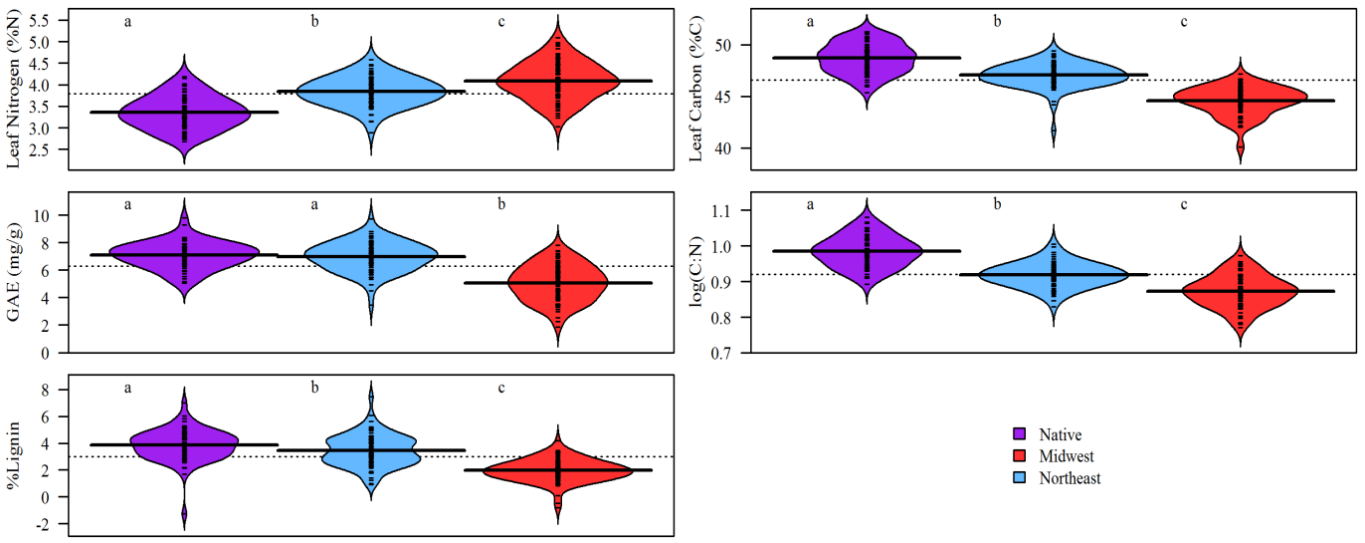

Mean Nitrogen (%)
Mean GAE(mg/g)
Mean Lignin(%)
Mean Carbon (%)
Mean C:N
Native
Midwest
Northeast

Figure 2.4


Leaf Nitrogen (%N)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
a
b
c
Leaf Carbon (%C)
50
45
40
a
b
c
GAE (mg/g)
10
8
6
4
2
0
a
a
b
log(C:N)
1.1
1.0
0.9
0.8
0.7
a
b
c
%Lignin
8
6
4
2
0
-2
a
b
c
Native
Midwest
Northeast

CHAPTER 3: SPECTROSCOPIC CHARACTERIZATIONS OF GENETICALLY-MEDIATED LEAF CHEMISTRIES IN BLACK LOCUST (*ROBINIA PSEUDOACACIA*)

Abstract Leaf chemical characteristics are critical to ecosystem function as they can drive major processes such as herbivory, nutrient cycling, and decomposition. While variation in leaf chemistry is important to ecosystem dynamics, traditional methods of analyzing leaf chemistries are often time consuming, costly, and destructive to plant tissues. Due to such limitations, studies have often been restricted to small spatial and temporal scales. The goal of my study is to characterize the potential predictive relationships between foliar, spectral, and genetic traits of black locust (*Robinia pseudoacacia* L.). I used a simple handheld USB-2000+VIS-IR-ES (340-1023 nm, 1.5 nm resolution) spectrophotometer (Ocean Optics, Dunedin, FL) to sample trees from three distinct regions that spanned over 1000 km of the North American range for this species. I collected one spectra measurement for each tree for up to 4 trees per site equaling a total of 255 spectral measurements. Spectra varied according to both plant genotype and, to a lesser extent, region. *In situ*-collected leaf spectra roughly estimated black locust leaf chemistries: % carbon (r^2=0.56, RMSE = 1.40), % nitrogen (r^2=0.47, RMSE=0.35), GAE (gallic acid equivalents; r^2=0.33, RMSE= 1.19), and % lignin (r^2=0.32, RMSE=1.08). Although absorption spectra varied by genotype, the ability of spectra to discriminate genotypes was generally weak due to the narrow range of chemical variation among genets. Consequently, while simple handheld spectrometers with

limited spectral ranges can provide rough estimates of important leaf chemistries, spectrophotometers that do not cover the full NIR spectra may not be sufficient for studies requiring accurate estimates of foliar chemistries.

INTRODUCTION

Plant chemistry traits are dominant drivers of ecosystem processes worldwide. Variations among plant chemical traits explain the majority of variation found in global decomposition and nutrient cycling (Zhang et al. 2008; Cornwell et al. 2008). Relative differences among commonly sampled chemistries such as leaf carbon, nitrogen, lignin, and phenolics are important contributing factors to key ecosystem processes such as decomposition and nutrient cycling (Talbot and Treseder 2012). For instance, lignin is the second most abundant naturally occurring polymer after cellulose and has important belowground effects (Hättenschwiler and Vitousek 2000). As a recalcitrant molecule, lignin decreases litter decomposition by protecting labile litter components from microbial decomposition (Berg and Staaf 1980; Melillo et al. 1982). Likewise, polyphenols are a diverse group of plant secondary metabolites that can have strong influences on nutrient cycling, especially for belowground systems (Hättenschwiler and Vitousek 2000). Often considered defense compounds, polyphenols can inhibit soil microbial communities. As soil microbial communities can control many rate limiting steps of nutrient cycling, microbial inhibition by polyphenolics can reduce nitrification and N mineralization rates, as well as retard decomposition (Baldwin et al. 1983; Horner et al. 1988). In some instances, polyphenolics can have a greater impact on belowground nutrient cycling than do other important chemistries such as leaf nitrogen and lignin (Palm and Sanchez 1990).

Plant chemical traits vary widely both across and within plant species. Across species, the impact of plant chemical traits on decomposition can be greater than that of abiotic factors (Cornwell et al. 2008). Within species, a growing number of studies indicate that intraspecific variation in plant chemistry can be as important to ecosystem function as is interspecific variation (Whitham et al. 2006; Lindroth et al. 2007; Hughes et al. 2008; Bailey et al. 2009). For instance, genetically-driven variation in plant chemistries have been shown to drive both the composition (Schweitzer et al. 2008), and functional roles (Madritch and Lindroth 2011), of belowground microbial communities. By altering belowground microbial structure and function through chemical inputs, plants can alter ecosystem nutrient cycling and availability. The ability to accurately predict patterns in both aboveground genetic and chemical patterns over large scales would provide a wealth of information on how nutrients are cycled through ecosystems.

While reflectance spectroscopy is a standard practice in the field of chemometrics, interest in its biological applications have gained traction, especially for estimating *in situ* leaf chemistries (Couture et al. 2013). Traditional methods of analyzing plant chemical characteristics are often time consuming, costly, and destructive to plant tissues (Gitelson et al. 2003; Blackburn 2007). In instances where plant tissues and cuttings must be transported to the lab for analysis, chemical changes can occur during transport and influence results. Moreover, destructive sampling can induce phytochemical changes, prohibiting repeated measurements across time. Due to such limitations, phytochemical-based research has often been restricted to small scale studies that are limited in spatial and/or temporal scales.

Advances in imaging spectroscopy offer potential solutions that could allow canopy foliar chemistries to be easily estimated across larger scales. Since spectral measurements can be taken quickly *in situ* using hand-held high resolution spectrometers, they may also provide useful preliminary information about the relative importance of different spectral bands to species chemistry. Pinpointing relevant bands may help to improve the results derived from other remote sensing platforms. Additionally, issues of leaf structural interference with chemical signals are pervasive in both leaf level and canopy level studies (Ustin 2013; Townsend et al. 2013). While dealing with issues of structural interference are no doubt challenging, improving chemistry characterizations from fresh *in situ* leaves may be more applicable to canopy and ecosystem measures than are laboratory-based measurements.

Chlorophyll absorbance meters are another widely used type of hand-held device that can be used for *in situ* leaf measurements. In contrast to handheld spectrophotometers which measure full spectrum leaf reflectance, chlorophyll absorbance meters estimate chlorophyll concentrations using leaf absorbance at wavelengths c. 660 nm (red) and c. 940 nm (NIR). Conceptually, chlorophyll absorbance meters measure chlorophyll because chlorophyll strongly absorbs red light, while the NIR serves as a reference spectrum to account for internal leaf structure (see Markwell et al. 1995). While chlorophyll absorbance meters can accurately estimate chlorophyll concentrations, they are limited in their ability to estimate other chemistry such as leaf nitrogen (Pinkard et al. 2006). Spectrophotometers have been shown to produce more accurate estimations of chlorophyll, likely due to the extensive amount of

information collected from the closely spaced wavelength bands (i.e. high spectral resolution ; Richardson et al. 2002).

The depth of spectral signatures affords them the potential to act as biological proxies to predict complex ecological relationships. For instance, Madritch et al. (2014) found that remotely sensed AVIRIS data could accurately predict aspen genotype and canopy chemistry. In addition, spectral signatures could be used to describe variation in the belowground functional roles of soil microbial communities (Madritch et al. 2014). Because leaf chemistry in black locust is likely in part driven by genetic variation, it may be possible to use a simple handheld spectrometer to estimate phylogenetic patterns at the ecosystem scale in black locust systems.

The goal of my study was to determine if an inexpensive and commonly available partial spectrum spectrometer (339-1022 nm) was capable of accurately describing common leaf chemistries. I used black locust as a model system because it has a large geographic range across a wide set of environmental conditions, and it has been shown to have genetically-mediated variation in leaf chemistries (Chapter 2). In addition, black locust is an invasive species that often grows in large genet patches within its invasive range, making it a suitable candidate for future remote sensing studies, as has been done with aspen (Madritch 2014).

METHODS

Leaf sample and spectral collections

I collected leaf samples in June of 2013 from three regions: 1) the Midwest region, located throughout southeastern Minnesota, southwestern Wisconsin, 2) the Northeast region northern, located throughout New Hampshire, Vermont, and the

southern tip of Maine, and 3) the native region, located throughout the Southeastern Appalachians, including the mountainous portions of northwestern North Carolina, western Virginia, and eastern West Virginia (Fig 1). Leaf reflectance was measured *in situ* using a high resolution USB-2000+VIS-IR-ES (340-1023 nm, 1.5 nm resolution) spectrophotometer (Ocean Optics, Dunedin, FL) in combination with a CI-710 Miniature Leaf spectrometer attachment (CID Bioscience, Camas, WA). The CI-710 leaf clip irradiates the leaf sample with light from a blue LED and an incandescent lamp, providing output in the visible-to-infrared range (400-1000nm range). The USB-2000+VIS-IR-ES detected reflectance from the abaxial side of one full-sun leaf per tree. Both spectral and tissue samples were collected from four trees within each sampling site. Leaves were stored in silica desiccant at 4°C before freeze-drying, and at -20°C afterward.

Leaf chemical analyses

I analyzed four foliar chemical traits known to vary by black locust genotype, including carbon (C), total nitrogen (N), lignin, and phenolics. Carbon and nitrogen were analyzed via combustion analysis using the Flash EA1112 elemental analyzer (Thermo Fisher Scientific, Waltham, MA). I used Gallic acid equivalents (GAE) as a proxy for total phenol estimation using the Folin-Ciocalteau reagent colorimetric assay with gallic acid standards (Cicco and Lattanzio 2011). Lignin concentrations were determined using a modified thioglycolic acid protocol (Suzuki et al. 2009) with kraft lignin (Sigma-Aldrich, St. Louis, MO) and purified barley as the standards and controls, respectively.

Genetic analysis

Genets were confirmed using eight species-specific microsatellite markers (Lian and Hogetsu 2002; Mishima et al. 2009). Leaf DNA was extracted using the DNeasy Plant Mini kit reagents (Qiagen, Venlo, Netherlands). M13-tailed forward primers were used for labeling according to the methods described by Schuelke (2000). A final volume of 15 µL containing 7 µL of 2X Master Mix (Promega Corp, Madison WI), 0.3 µM M13-tailed forward primer, 0.3 µM M13 fluorescent dye (FAM, VIC, NED, PET), 0.6 µM reverse primer and 5-10 ng of DNA template was used for the PCR reactions. A touchdown PCR protocol, as described by Mishima et al. (2009), was performed using a Mastercycler Nexus (Eppendorf, Hamburg, Germany). Resulting PCR products were multiplexed with the GeneScan Liz 600 size standard and HiDi formamide (Applied Biosystems, Foster City, CA). Multiplexed samples were separated using an ABI 3730 sequencer (Applied Biosystems, Foster City, CA) at the Georgia Genomics Facility (Athens, GA). Four quality control samples were included per plate to allow for sequencing and scoring error detection and correction.

Statistical analyses

Variation in chemistry

To account for the hierarchical data structure and non-independence of genet replicates, I used linear mixed models to determine the influence of region and genet identity on individual leaf chemistries. Diameter at breast height (DBH) for each tree was initially included in the model as a proxy for tree age, but it did not explain any significant variation in leaf chemistries and was therefore removed. I ran all linear mixed models using the lmer function in the R package lme4 (Bates et al. 2014). I used

additional R packages to generate Type III ANOVA tables (car) and calculate AICc (AICcmodavg; Fox and Weisberg 2011; Mazerolle 2013). For each leaf chemistry, I employed both a reduced model, with no fixed effect and genet identity as a random effect, and a full model, with region as a fixed effect and genet identity as a random effect. I used the R package MuMI to calculate the marginal and conditional R^2 which measure the amount of variation explained by the fixed effects and the full model, respectively (Nakagawa and Schielzeth 2013). Regional differences in leaf chemistry between regions were analyzed using a one-way ANOVA with a Tukey's post hoc means comparison.

Genetic analyses

In order to confirm genet membership, I genotyped all sampled trees with eight species-specific microsatellite markers (Lian and Hogetsu 2002; Mishima et al. 2009). Chromatograms were scored using Peak Scanner software version 2.0 (Life Technologies, Carlsbad, CA). I found 20, 25, and 24 distinct genets in the Native, Midwest, and Northeast regions, respectively.

Genet spectral variation

To quantify differences in spectral variation among regions and among genets within regions, Nonmetric Multidimensional Scaling (NMS) using Sørensen-Bray distance and Multiple Response Permutation Procedure (MRPP) were performed. NMS was useful for determining the amount of distance among spectra explained by genet identity, whereas MRPP was used to determine whether leaf spectra agree within genets. The resulting A and T statistics from the MRPP are measures of the within group homogeneity and the relative differences among groups, respectively. The A statistic is

scaled from 0-1 with 0 being no agreement and 1 being complete agreement. The T statistics is relative, with more negative values indicating greater differences between groups. NMS and MRPP analyses were performed using PC-ORD v.6.0 (MjM Software Design, Gleneden Beach, OR).

Chemical predictive capabilities

Partial Least Squares regressions (PLSR) were used to create a predictive model linking spectral signature to leaf chemistry. PLS is a method commonly used in chemometrics and has been more recently applied to biological studies (Bolster et al. 1996; Asner and Martin 2008; Asner et al. 2011; Serbin et al. 2012; Couture et al. 2013).The number of latent variable components was determined iteratively through the reduction of the root mean Predicted Residual Sum of Squares (PRESS) statistics followed by the reduction in Root Mean Square Error (RMSE, Serbin et al. 2012). An initial null model (i.e. all spectral bands) was used to calculate the VIP (Variable Importance) of each band (calculated in JMP Pro 10, SAS Institute, Kary, NC). Spectral bands with a VIP score >1 were removed for the final model. Data were partitioned into training (70%) and testing sets (30%) for cross-validation and 100 randomized model subsets were permutated. PLS analyses were performed using the R package 'pls' (Mevik and Wehrens 2007).

Genet discrimination abilities

A PLS discrimination analysis (PLS-DA) was used to predict genet membership based on variations among leaf spectral signatures. PLS methods are useful for dealing with highly collinear data, such as among spectra bands or leaf chemistries (Martens and Jensen 1983; Wold and Sjostrom 2001). In PLS analyses, both explanatory and

response variables are projected into latent variable structures that maximize their covariance. For a PLS discrimination analysis (PLS-DA), the response variable is categorical (e.g. genet). For both the spectral and chemical discrimination analyses, k-1 components were used where k is the number of classes or genets. For model calibration, the data were partitioned into training (70%) and testing sets (30%) for cross-validation. Randomized model subsets were permutated 100 times and the results were averaged. PLS-DA analyses were performed using the R (R Core Team 2014) package 'caret' (Kuhn 2008).

RESULTS

Chemical and genotypic variation

Leaf chemistries varied among both regions and genets. Among regions, nitrogen ranged from 3.36-4.09%, carbon from 44.56-48.69%, GAE from 5.05-7.08 mg/g, and lignin from 1.96-3.85% (Table 3.1, Figure 2.1). The ranges of variation in leaf chemistries within regions were also significant, but relatively low (Table 3.1). The amount of variation in leaf chemistry explained by genet effects ranged between 9-30% (See Table 2.3).

Genet spectral variation

Spectral signatures were unique among genets. The A statistic, representing the degree of within genet spectral homogeneity, ranged from 0.52-0.67 within each region and was 0.66 across all regions (Table 3.2). Additionally, to a lesser extent, spectra were more similar within regions than across regions (A=0.19). While genets have unique spectral signatures, the NMS analysis showed a high degree of overlap between genets (Figure 3.1).

Chemical predictive capabilities

The PLS regression models created for the four leaf chemistries considered here varied in their predictive capabilities. The R^2 between the predicted and actual values for the validation models ranged from 0.32-0.59 (Table 3.3, Figure 3.2). For lignin, four latent variable components explained 98.46% of the variability in spectra but only 35.14% of the variability in lignin concentration. Leaf carbon was best summarized by 10 latent variable components explaining 99.22% of the variability in spectra and 98.49% of the variability in %C. GAE was summarized using 9 latent variables components explaining 99.16% of the variation in spectra and 94.90% of the variation in GAE concentration. Nitrogen was summarized using 11 latent variable components explaining 99.19% and 99.40% of the variability in spectra and %N, respectively. Lastly, C:N was summarized using 8 latent variable components that explained 98.97% of the variation in the spectra and 92.48% of the variation in the ratio. The VIP values indicated that wavelengths between approximately 400-500 nm and 600-750 nm contributed the most to the models (Figure 3.3).

Genet discrimination

The levels of agreement between the predicted genet membership and the actual membership based on molecular data were low for both the leaf chemistry and spectral discrimination analyses as indicated by the mean Kappa statistics (Table 3.4). For the spectral discrimination analysis, membership agreement ranged from 5-13%, with regional models performing slightly better than the overall model. Membership agreement for the leaf chemistry discrimination model ranged from 2-11%, with the weakest agreement in the Northeast region.

DISCUSSION

My research demonstrates that black locust genotypes have unique chemical and spectral characteristics. Although the ranges of variation in leaf chemistries were low, variation among genotypes may still play an important role in belowground systems. The impacts of chemical variation on belowground processes may be even more profound in regions with invasive populations of black locust, as black locust is known to impact soil carbon and nitrogen dynamics (Malcolm et al. 2008; De Marco et al. 2012).

Based on my previous work that demonstrates genet identity explains much of the variation in leaf chemistry (Chapter 2), it is not surprising that genets had unique spectral signatures. The results of the MRPP showed particularly strong differences between genet spectral signatures. However, as evident from the NMS analysis, there is still a high degree of overlap between spectral signatures, which is consistent with what would be expected for chemistries with low amounts of variation. Overall, the PLS models were able to roughly estimate the four functionally important leaf chemistries. Interestingly, carbon, nitrogen, and their ratio had the best fit PLS models in terms of their predictive abilities. Carbon and nitrogen also had the highest and lowest variation in concentrations, respectively. For carbon, the high variation in concentration may have contributed to a better fit that increased the predictive power of the model. Nitrogen likely performed well due to the contributions of known chlorophyll and nitrogen associated spectral bands, such as between 690 nm and 740 nm, included in the model (Curran et al. 1990). Additionally, predictive ability of the lignin PLS model was likely limited due to the missing latter portions of the NIR spectrum. Other studies

have shown that wavelength ranges associated with lignin absorption features primarily occur above 1120 nm (Elvidge 1990; Fourty et al. 1996).

In other systems, PLS methods have accurately characterized leaf chemistries based on spectra taken from fresh leaf samples. For instance, Couture et al. (2013) were able to estimate concentrations of cardenolide in *Asclepias syricaca* L. with an average R^2 and RMSE of 0.85 and 0.22µg/mg, respectively. In comparison, the resulting PLS models used in my study performed quite poorly. Based solely on R^2 values, the leaf nitrogen PLS model performed little better than the results found by Pinkard et al. (2006) using a chlorophyll absorbance meter (both with an R^2 of 0.47). The limited accuracy of my results is likely due to biological, experimental, and instrumental limitations. Additionally, low variation in leaf chemistries, particularly in leaf nitrogen, may limit the predictive ability of the regression models. A low number of replicates per genotype compared to other studies could weaken the models as well (Madritch et al. 2014). In addition to the biological system and sampling design used here, the spectrophotometer used in this study only covered a small portion of the NIR (near infrared) spectrum (800-1022nm). The excluded portion of the NIR range (1022-2500nm) is likely essential for creating robust regression models.

While my study clearly demonstrates differences in spectral signatures among genets, the spectral range employed was not sufficient to accurately discriminate among genets. One interesting outcome of the PLS-DA models was the trend showing that regional models performed better than the full model. This pattern may suggest that spectral variations were unique to regions, which may explain why regional models perform slightly better than did the full model, despite being trained with fewer

samples. Use of a spectroradiometer that covers the full visible and NIR (~380-2500 nm) may provide clearer results, but such instruments can be prohibitively expensive. For instance, using PLS-DA analyses, Madritch et al. (2014) found upwards of 89% agreement between actual and predicted genet classification. Researchers should be aware that spectrometers with limited spectral capability may yield equally limited biologically relevant information.

My research demonstrated that there are unique spectral and chemical differences among genets of black locust. Understanding how chemical and spectral differences translate into changes in ecosystem function is the essential next step to gain a better understanding of how black locust can alter its environment. Further exploring how regional differences in black locust traits can alter belowground processes may provide insight into which traits have allowed black locust to become one of the most widely distributed trees worldwide. Additionally, rough estimates of important chemical traits, particularly leaf carbon and nitrogen, can be made using a partial spectrum hand-held spectrometer. These findings suggest that improving models by analyzing additionally leaf chemistries or using full spectrum analyses could potentially lead to better discrimination among genotypes of black locust.

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TABLES

Table 3.1 Average leaf chemistry concentrations ± (se) and range between minimum and maximum chemical concentrations.

Leaf Chemistry	Native (n=72)		Midwest (n=95)		Northeast (n=88)	
	Mean (se)	Range	Mean (se)	Range	Mean (se)	Range
Nitrogen (%)	3.36 (0.04)	1.50	4.09 (0.04)	2.08	3.85 (0.04)	1.70
Carbon (%)	48.69 (0.16)	5.86	44.56 (0.14)	7.03	47.04 (0.14)	7.70
GAE (mg/g)	7.08 (0.13)	4.76	5.05 (0.12)	5.96	6.98 (0.12)	6.27
Lignin (%)	3.85 (0.12)	5.29	1.96 (0.11)	4.97	3.44 (0.11)	6.51

Table 3.2 Results of a multi-response permutation procedure (MRPP) using Sørensen-Bray Distance for spectra among regions, all genets, and genets within region. The A and T statistic measure the within group homogeneity and the relative differences among groups, respectively. The A statistic is scaled from 0-1 with 0 random change agreement and 1 being complete agreement. The T statistic is relative, with more negative values indicating greater differences between groups. The p-value indicates the significance of the A statisitic.

	T	A	*p*
All Regions			
Region	-52.80	0.19	<0.0001
Genet	-23.49	0.66	<0.0001
Native			
Genet	-12.49	0.67	<0.0001
Midwest			
Genet	-13.38	0.52	<0.0001
Northeast			
Genet	-12.58	0.54	<0.0001

Table 3.3 Partial Least Squares regression results for leaf chemistry.

Leaf Chemistry	R^2	RMSE
Nitrogen (%)	0.47 (0.10)	0.35 (0.03)
Carbon (%)	0.56 (0.07)	1.40 (0.13)
GAE (mg/g)	0.33 (0.08)	1.19 (0.08)
Lignin (%)	0.32 (0.07)	1.08 (0.09)
C:N	0.59 (0.09)	1.22 (0.12)

Table 3.4 Partial Least Squares discrimination analysis of black locust genets by spectral signature and leaf chemistry. The Kappa statistic is the average of 100 permutations. The Kappa statistic indicates the level of agreement between the predicted and actual genet membership. A Kappa value of 1 indicates complete agreement, while a Kappa value of 0 indicates no agreement.

	n genotype	n trees	Mean Kappa Statistic
Spectra			
All Regions	81	283	0.06
Native	26	84	0.09
Midwest	28	101	0.12
Northeast	27	98	0.14
Leaf Chemistry			
All Regions	66	242	0.04
Native	20	71	0.12
Midwest	25	90	0.09
Northeast	21	81	0.03

FIGURE LEGEND

Figure 3.1 Non-metric Multidimensional Scaling of the Midwest, Northeast and native range genets.

Figure 3.2 Partial Least Squares validation regressions for actual vs. predicted chemistries.

Figure 3.3 Smoothed spline fit of variable importance scores by wavelength (smoothing parameter =0.35). The dotted horizontal line shows the cut-off point for wavelengths included in the PLS models. All values for wavelengths below 1.0 were removed for the Partial Least Squares regression model.

FIGURES

Figure 3.1

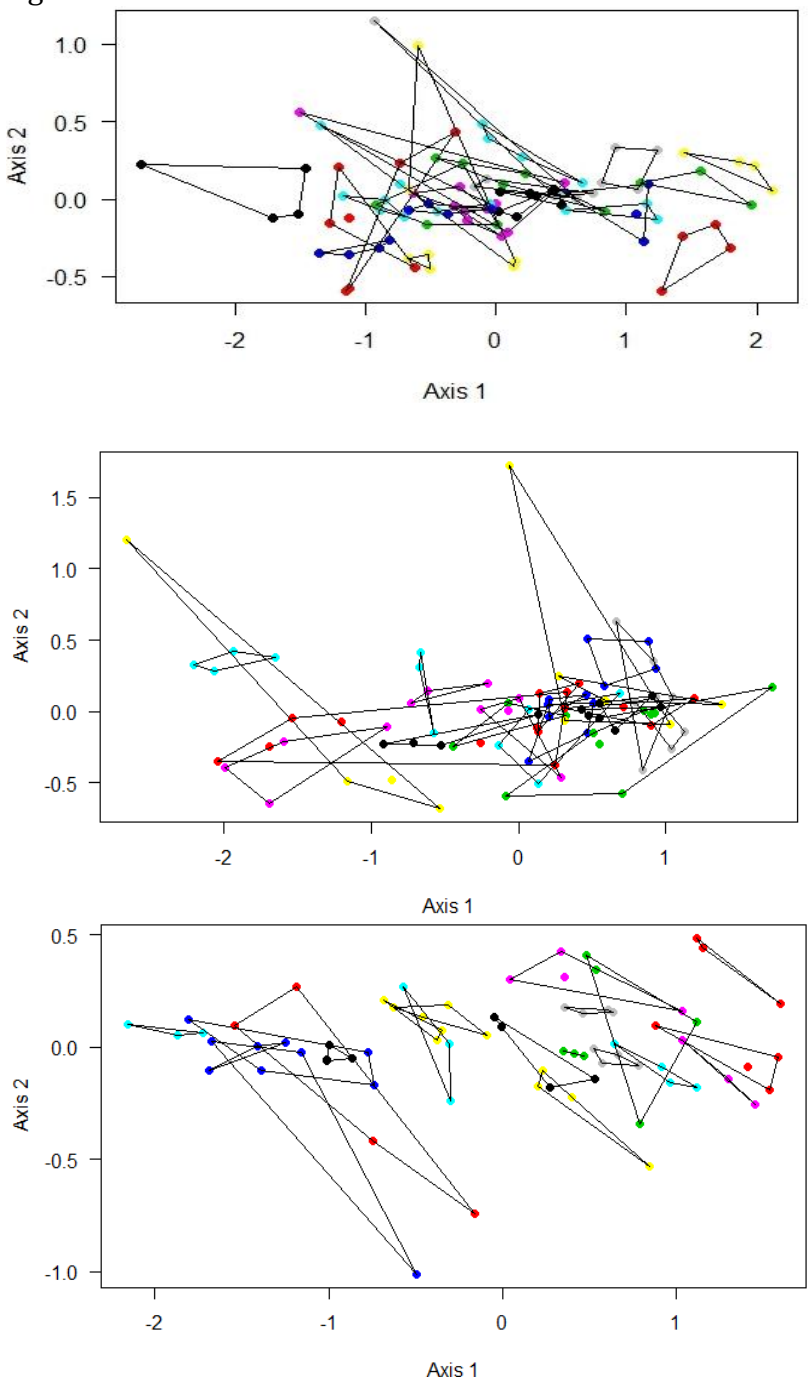


Figure 3.2

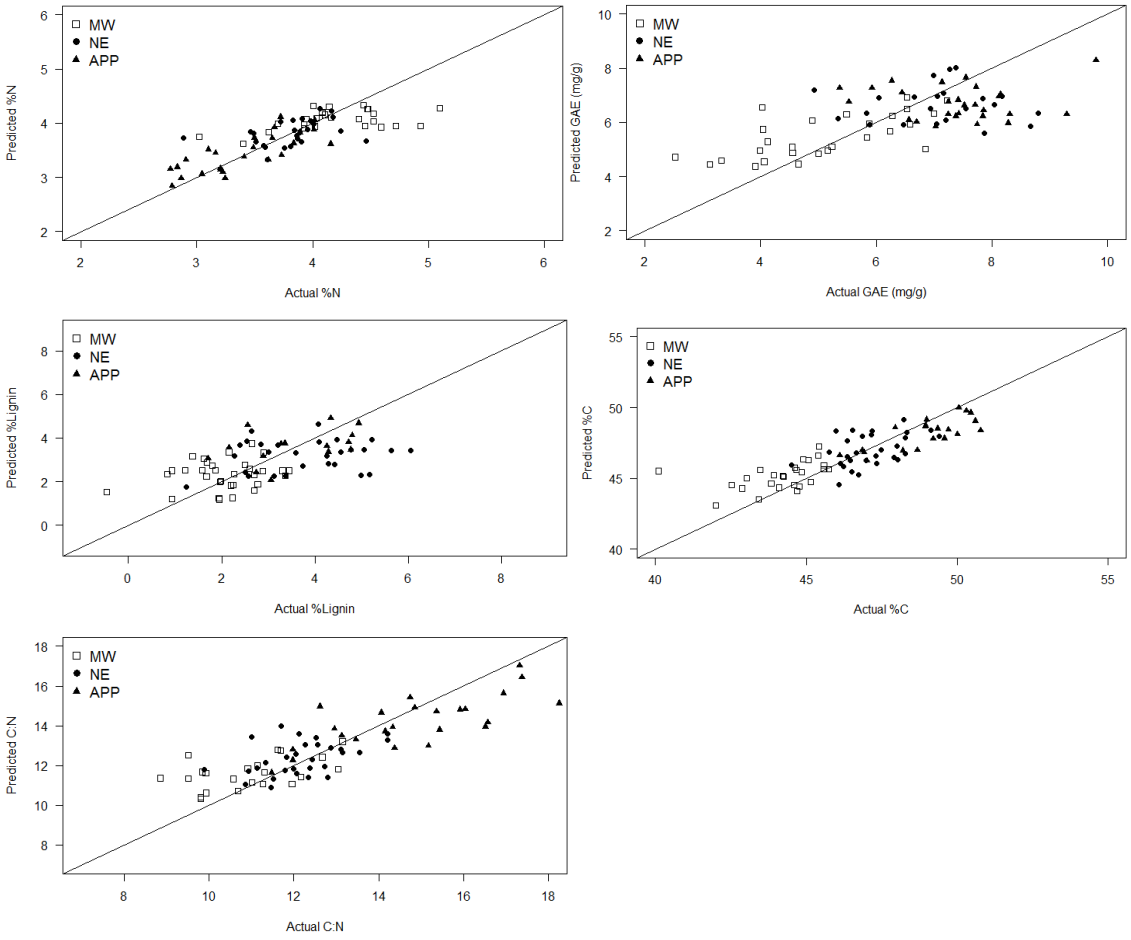
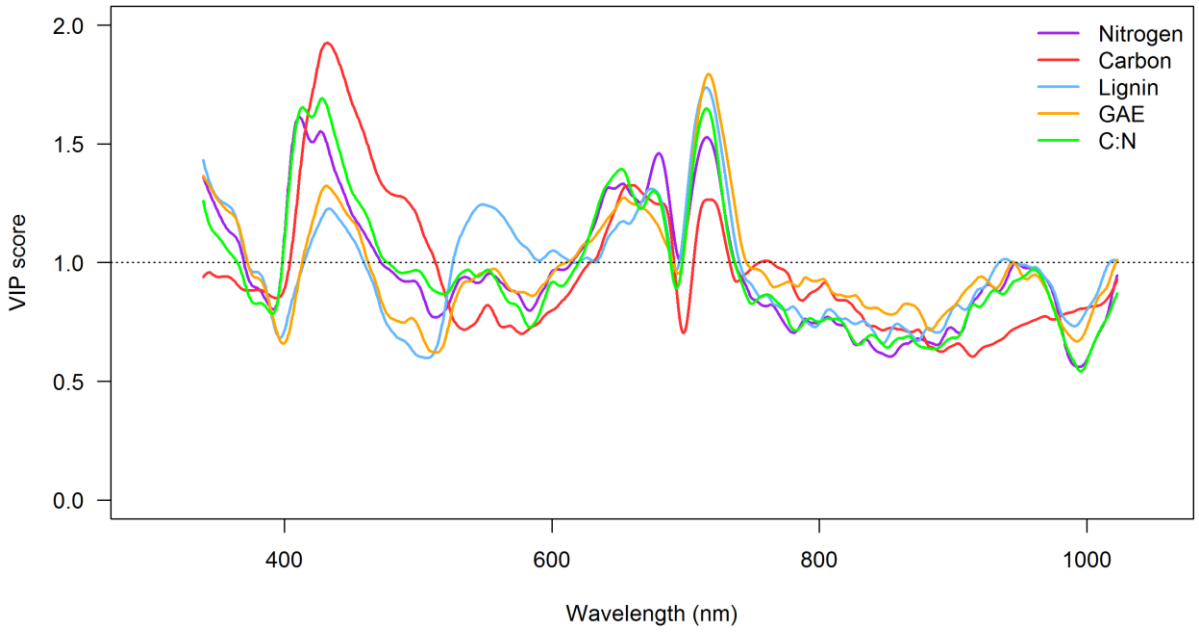

MW
NE
APP
Predicted %N
Actual %N
Predicted GAE (mg/g)
Actual GAE (mg/g)
Predicted %Lignin
Actual %Lignin
Predicted %C
Actual %C
Predicted C:N
Actual C:N

Figure 3.3


2.0
1.5
1.0
0.5
0.0
VIP score
400
600
800
1000
Wavelength (nm)
Nitrogen
Carbon
Lignin
GAE
C:N

Vita

Cameron Kelly Houser was born in Boiling Springs, North Carolina, to Dan and Lisa Houser. She graduated from the North Carolina School of Science and Mathematics in May 2007. The following autumn, she entered the University of North Carolina at Chapel Hill to study Environmental Science, and in May 2011 she was awarded the Bachelor of Science degree. In the fall of 2012, she accepted a graduate assistantship in Biology at Appalachian State University and began study toward a Master of Science degree. The M.S. was awarded in August 2014. In August 2014, Ms. Houser commenced work toward her Ph.D. in Remote Sensing at Virginia Polytechnic Institute and State University.