

Genetically-mediated variation in above and belowground chemistries in invasive and native black locust (*Robinia pseudoacacia* L.) ecosystems

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**Abstract** Black locust (*Robinia pseudoacacia* L.) is one of the few examples of an intra-continently invasive tree.

As such, black locust ecosystems provide a unique opportunity to study biological invasion in the absence of major geographical barriers. As a nitrogen-fixing species, black locust has the potential to strongly influence ecosystems.

In addition to increasing nitrogen inputs belowground, leaf chemistry traits of black locust may also impact ecosystem processes and nutrient cycling. We quantified the influence of region and genetics on variation in native and invasive leaf chemistries (carbon, nitrogen, total phenolics, and lignin) and their relationship to belowground nitrogen pools (soil  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ). Using eight microsatellite markers, we identified genets with multiple ramets in the native Appalachian region (N=20), and two invaded regions in the Northeast (N=22) and Midwest (N=25) United States. All regions exhibited high genetic diversity and little interregional genetic differentiation (1-3%). Up to 30% of the variation in leaf chemistries and 43% of the variation in soil nitrogen were genetically-driven. Due to strong genetic influence and low variation in leaf chemistries, correlations between leaf and soil chemistries were weak. However, the presence of black locust increased soil  $\text{NO}_3^-$  by 177% in the Northeast, 39% in the Midwest, and 144% in the Native region. Our findings suggest that black locust uniquely alters invaded regions by increasing soil nitrogen pools and that the degree of soil nitrogen increase varies by genotype. These findings emphasize the important role genetic variation plays in determining how invasive species alter ecosystems.

#### **Keywords**

Nitrogen-fixation, species invasion, soil nitrogen, genetic diversity, ecosystem processes

#### **Introduction**

Biological invasions can have ecosystem-level effects when invading species introduce a new biological process such as nitrogen fixation to 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 overall nitrogen (N) availability (Vitousek and Walker 1989). Generally, the fates of this newly available nitrogen (as  $\text{NH}_4^+$ ) are either be

taken up directly by roots, or transformed into  $\text{NO}_2^-$  and  $\text{NO}_3^-$ .  $\text{NO}_x^-$  not sequestered by plants or microbes can be leached from soils and enter into waterways, impacting both terrestrial and aquatic ecosystems (Hughes and Denslow 2005; Wiegner et al. 2013). For instance, another N-fixing invasive tree *Falcataria moluccana* changes the composition of Hawaiian forests, increases soil nutrients, and increases stream nitrate levels by up to 600% (Hughes and Denslow 2005; Wiegner et al. 2013). Increasing nitrogen availability in N-limited ecosystems can increase soil fertility and net primary production (NPP; 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 other nonnative species (Simberloff and Holle 1999; Von Holle et al. 2006). Competition for resources can disrupt native plant communities, especially in communities without a native N-fixing species (Simberloff and Holle 1999).

Native to the southern and central Appalachians and the Ozarks, black locust (*Robinia pseudoacacia* L.) is now 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 black locust's extensive geographic range and N-fixing capabilities, it represents an important invasive species that could have large ecosystem-level impacts. For example, after disturbances such as logging, black locust can grow rapidly and return N soil pools to pre-disturbance levels in less than four years (Boring and Swank 1984). Considering that approximately 10% of all invasive plant species in North America have nitrogen-fixing capabilities (Ehrenfeld 2003), understanding the mechanisms that lead to black locust's success as an invader is an important step for assessing the invasive potential within this particular N-fixing functional group.

Genetics play an important role in determining the invasive potential and success of a species. High genetic diversity may increase invasiveness by affording species greater flexibility in their response to novel environmental conditions (Sakai et al. 2001; Allendorf and Lundquist 2003). Two commonly invoked mechanisms of invasion, evolution of increased competitive ability and optimal defense theory, require genetic variability to allow an adaptive response to a novel environment (McKey 1974; McKey 1979; Blossey and Notzold 1995). Several studies 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 affords black locust the adaptive potential to expand its range and compete with native species.

Invasive species can alter ecosystem function through variation in litter chemistry by affecting detrital pathways, plant growth, and rates of nutrient cycling (reviewed by Vitousek 1990). For instance, low concentrations of defensive compounds such as polyphenolics can lead to fast decomposition and nutrient cycling (Hättenschwiler and Vitousek 2000). One difficulty in predicting the impact of an invasive species is that post-invasion shifts in functional traits can lead to changes in litter chemistry (Mooney and Cleland 2001; Lee 2002; Pigliucci 2005). For instance, invasive plants may shift resources from defense to growth, and such shifts in allocation would likely result in a reduction of defensive compounds (Keane and Crawley 2002; Cappuccino and Carpenter 2005; but see Agrawal and Kotanen 2003). Such is the case for the plant *Silene latifolia*, a North American invasive introduced from Europe whose invasive populations evolved to allocate plant resources to growth rather than to defensive compounds (Blair and Wolfe 2004). Aggressive invaders, such as black locust, may be more likely to experience enemy release (Mitchell and Power 2003; Carpenter and Cappuccino 2005; Cappuccino and Carpenter 2005). Invasive stands of black locust often facilitate higher understory nonnative richness and abundance than do paired native stands (Von Holle et al. 2006), highlighting the potential of black locust to alter nutrient cycling. This pattern of nonnative facilitation may be driven in part by increased nutrient cycling combined with lowered defensive compounds. The combined effects of reduced defensive compounds and increased nitrogen availability from black locust invasion likely affect belowground processes resulting in changes to ecosystem function.

Leaf chemistry is important to ecosystem-level processes and can vary widely by plant 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). 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.

Here, we compare genetic and chemical characteristics of black locust between the native and North American invasive ranges *in situ*. Our goals were to 1) analyze the degree of genetic differentiation and genetic structure among and within regions, 2) determine whether 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 high concentration of phenolics and C:N ratios are expected to be higher in the native region than in the invaded 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, we selected locations that fell within the Environmental Protection Agency's (EPA) level II ecoregions (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*

We collected leaf and soil samples in the summer of 2013 from the three regions. Within each region, leaf tissue samples were collected from four individual trees at each of 30 sampling locations (4 trees x 30 sites x 3 regions = 360 tree samples). 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 diameter at breast height (DBH) greater than 5 cm. Leaves were stored in silica desiccant at 4°C before freeze-drying and subsequent storage at -20°C before further analysis.

From beneath each tree sampled for foliage, we collected a composite soil sample by pooling three 15 cm deep 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. All leaf and soil samples were ground using a ball mill before subsequent analyses below.

#### *DNA extraction and Microsatellite analyses*

Genomic DNA was extracted from the leaves of each individual tree using DNeasy Plant Mini kits (Qiagen, Venlo, Netherlands). For genotyping analysis, we used eight pairs of co-dominant, species-specific, microsatellite markers (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  $\mu$ L containing 7  $\mu$ L of 2X Master Mix (Promega Corp, Madison WI), 0.3  $\mu$ M M13-tailed forward primer, 0.3  $\mu$ M M13 fluorescent dye (FAM, VIC, NED, PET), 0.6  $\mu$ M reverse primer, and 5-10 ng of DNA template. Reactions were performed using the touchdown PCR protocol described by Mishima et al. (2009), and multiplexed with the GeneScan Liz 600 size standard and HiDi formamide (Applied Biosystems, Foster City, CA). Samples 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*

We analyzed leaf samples for total carbon (C), total nitrogen (N), lignin, and phenolics. Carbon and nitrogen were analyzed via combustion analysis with a Flash EA1112 elemental analyzer (Thermo Fisher Scientific, Waltham, MA). Total phenolics were measured as gallic acid equivalents (GAE) using the Folin-Ciocalteu 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) using kraft lignin (Sigma-Aldrich, St. Louis, MO) and purified barley as standards and controls, respectively. Soil ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) were extracted with 2M KCl. Soil extracts were analyzed for ammonium using the indophenol blue method (Mulvaney 1996). Soil extract nitrate concentrations were determined by reducing nitrate to nitrite using vanadium (III) chloride, followed by colorimetric analysis with Griess' Reagent (Doane and Horwáth 2003).

#### *Genetic analyses*

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 genetic 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 (HWE) 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 ( $F_{st}$ ) and Goodman's Estimator ( $R_{st}$ ). Both statistics of differentiation were included because while Wright's F statistic is widely used,  $R_{st}$  assumes a step-wise mutational model that is 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  $F_{st}$  and  $R_{st}$ .

#### *Genetic structuring*

A Principle Coordinates Analysis (PCoA) implemented in PopGenReport was used first to explore the range-wide structure. STRUCTURE (v.2.3) was used to identify the genetic structure of black locust across regions. STRUCTURE 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. We 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  $\Delta K$  was used to identify the most likely number of clusters. All  $\Delta K$  values were calculated using the program Structure Harvester (Earl and vonHoldt 2011). STRUCTURE outputs were corrected for issues of label-switching and multimodality using the program CLUMPP (Jakobsson and Rosenberg 2007).

#### *Above and belowground chemistry*

As a manipulative large-scale study within the continental native and invasive range was impractical, we 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, our 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 ensure dominance of genet litter inputs into belowground systems, we analyzed data from sites that had genets represented by three or four ramets and excluded those with only one or two ramets per genet. To isolate the effects

of black locust on belowground soil chemistries, we subtracted the average chemistry of four paired control soil samples from the individual soil chemistries sampled below each genet (henceforth referred to as  $\Delta\text{NO}_3$  and  $\Delta\text{NH}_4$ ).

To account for the hierarchical data structure and non-independence of genet replicates, we used linear mixed models to explain the influence of native status, region, and genet identity on individual soil and leaf chemistries. We 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. We ran all models using the lmer function in the R package lme4 (Bates et al. 2014) and 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), we 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), we 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. To estimate confidence intervals for the  $R^2$  values, we bootstrapped the models for 10,000 permutations. 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, we scored an average of 141 for each of the eight microsatellite loci (Table 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) (Table 1). There were no significant differences in frequency-based indices among the regions (Table 2). However, the Native region had the highest allelic diversity and number of private alleles (alleles unique to the region; Table 2). For all regions, observed heterozygosity was lower than was expected based on HWE (Table 2). Positive fixation indices suggested that excess homozygosity was possibly the result of inbreeding.

Some genetic differentiation did occur among the three regions (Fig. 2). The average differentiation across all regions based on  $F_{st}$  and  $R_{st}$  values were 1.8% and 3.2%, respectively. Regional pairwise  $F_{st}$  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, variation among genets explained 9-30% of the variation in leaf chemistry (Table 3, Fig. 3). Additionally, average chemistries were different among regions (Fig. 5). 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 nitrogen, genet identity explained a greater proportion of the variation than did region (Table 4). After soil extracts were normalized by control sites ( $\Delta\text{NH}_4$  and  $\Delta\text{NO}_3$ ), 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  $\text{NO}_3^-$  for each region compared to the average control  $\text{NO}_3^-$  (Table 5). The presence of black locust increases soil  $\text{NO}_3^-$  by 177% in the Northeast, 144% in the Native region, and 39% in the Midwest. Soil  $\text{NH}_4^+$  did not increase beneath black locust across all sites, but instead decreased by 14% in the Midwest region (Table 5). Despite significant genet effects that explained variation in both leaf and soil chemistries, the above and belowground chemistries were not well correlated with each other (Table 6).

#### **Discussion**

The impacts of invasive species largely depend on the ecological context within which the invasion occurs (Ehrenfeld 2003). Many studies show that invasive plants can vary in their degree of impact across environments and even across sites (reviewed by Ehrenfeld 2003). Our work demonstrates that in black locust systems, a portion of the variation in leaf and soil chemistry is driven by genetics. These findings highlight how genetic mechanisms can have impacts that extend beyond the population level and resonate at the community and ecosystem levels

(Whitham et al. 2006; Schweitzer et al. 2008). In the context of invasion, it may be difficult to establish generalized ecosystem impacts for widespread and genetically diverse species such as black locust without consideration of genetically-driven variation in chemical traits.

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 among regions (1-3%) and high genetic diversity within/across regions indicates gene flow among regions, most likely as the result of multiple introductions. High gene flow is further supported by the lack of spatial genetic structure between or within regions. Despite high observed heterozygosity ( $H_o$ ) across all regions, heterozygosity is lower than what was expected ( $H_e$ ) based on allele frequencies, which suggests there may be low levels of inbreeding or genetic substructuring within regions (Surles et al. 1990). High seed abortion and lowered fitness caused by inbreeding depression may act as a barrier to self-fertilization in black locust (Yuan et al. 2013). Barriers to inbreeding and inbreeding depression are mechanisms through which black locust populations could maintain both high genetic diversity and low levels of inbreeding.

Our data indicate that much of the variation in leaf chemistry is explained by genet identity. Differences in leaf quality (C:N, lignin, and GAE) between native and invaded regions 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 (Fig.3). 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 miner (*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). While the regional herbivory pressure exerted by specific herbivores on black locust remain unknown, the pattern still remains that invasive population have less defensive compounds than do native populations. Decreased herbivory pressure outside of the native range 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 mineral nitrogen, as demonstrated by  $\Delta NH_4$  and  $\Delta NO_3$  data (Fig. 4). The influence of genet in belowground nitrogen availability is notable considering

the clonal population structure of black locust. These results suggest that there is a patchwork-patterned impact of black locust across invaded ecosystems. Overall, the net effect of black locust invasion increased soil  $\text{NO}_3^-$  the most in the Northeast (177%) and Native (144%) regions. Soil  $\text{NO}_3^-$  increased by a lesser degree (39%) in the Midwest region. Differences in rates of anthropogenic N deposition among the regions does not explain differences in black locust-induced increases in soil nitrate. The Midwest and Native regions have similar N deposition rates of ~4-6 kg/ha per year, and the Northeast region has lower rates of ~1-2 kg/ha (National Atmospheric Deposition Program, <http://nadp.sws.uiuc.edu/>). The relatively small increase in soil  $\text{NO}_3^-$  associated with black locust colonization (?) in the Midwest was potentially due to the higher control soil  $\text{NO}_3^-$  levels which were 88-263% higher than for the other two regions. High levels of soil nitrate can inhibit nodule formation and thus, N-fixation in black locust (Boring and Swank 1984; Röhm and Werner 1991). The regional average soil  $\text{NH}_4^+$  did not vary from the controls in the Northeast or the Native regions, likely because  $\text{NH}_4^+$  was quickly immobilized by plants and microbes. Nonetheless, the large impact of black locust on variation in belowground nitrogen pools is in part driven by genotype.

Leaf and soil chemistries were weakly correlated suggesting that variation in litter inputs from black locust did not drive variation belowground  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations. The effect of variation in leaf chemistry on belowground chemistry was likely obscured, in part, by variation in belowground nitrogen fixation in the roots. While leaf nitrogen varied by genotype, the range of N variation was relatively small (3-4%). Additionally, 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. 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 availability, therefore resulting in similar N concentrations across trees. While leaf N variation was low, leaf lignin concentrations varied 5-fold and may have influence belowground processes that we did not measure here.

Our 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 plant genotype. Such alterations in ecosystem function are consistent with other studies of invasive nitrogen fixing trees (e.g. Vitousek and Walker 1989; Hughes and Denslow 2005, etc.). 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). 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. Understanding the role that genetic identity contributes to such environmental changes could help elucidate how changes occur across local and regional scales and deserves further attention in this system, as well as other N-fixing invasive species.

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#### LITERATURE CITED

- Adamack A, Gruber B (2014) PopGenReport: simplifying basic population genetic analyses in R. *Methods Ecol Evol*. doi: 10.1111/2041-210X.12158
- Agrawal AA, Kotanen PM (2003) Herbivores and the success of exotic plants: a phylogenetically controlled experiment. *Ecol Lett* 6:712–715. doi: 10.1046/j.1461-0248.2003.00498.x
- Allendorf F, Lundquist L (2003) Introduction: population biology, evolution, and control of invasive species. *Conserv Biol* 17:24–30
- Athey LA, Connor EF (1989) The relationship between leaf nitrogen content and feeding by *Odontota dorsalis* Thun. on *Robinia pseudoacacia* L. *Oecologia* 79:390–394. doi: 10.1007/BF00384319
- Bates D, Maechler M, Bolker B, Walker S (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-6
- Blair A, Wolfe L (2004) The evolution of an invasive plant: an experimental study with *Silene latifolia*. *Ecology* 85:3035–3042
- Blossey B, Notzold R (1995) Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. *J Ecol* 83:887–889. doi: 10.2307/2261425
- Boring L, Swank W (1984) The role of black locust (*Robinia pseudoacacia*) in forest succession. *J Ecol* 72:749–766
- Cadotte MW, Borer ET, Seabloom EW, et al. (2010) Phylogenetic patterns differ for native and exotic plant communities across a richness gradient in Northern California. *Divers Distrib* 16:892–901. doi: 10.1111/j.1472-4642.2010.00700.x
- Cappuccino N, Carpenter D (2005) Invasive exotic plants suffer less herbivory than non-invasive exotic plants. *Biol Lett* 1:435–8. doi: 10.1098/rsbl.2005.0341
- Carpenter D, Cappuccino N (2005) Herbivory, time since introduction and the invasiveness of exotic plants. *J Ecol* 93:315–321. doi: 10.1111/j.1365-2745.2005.00973.x
- Chang C, Bongarten B, Hamrick J (1998) Genetic structure of natural populations of black locust (*Robinia pseudoacacia* L.) at Coweeta, North Carolina. *J Plant Res* 17–24
- Cicco N, Lattanzio V (2011) Influence of initial carbonate concentration on the Folin-Ciocalteu micro-method for the Determination of Phenolics with Low Concentration in the Presence. *Am J Anal Chem* 2:832–839. doi: 10.4236/ajac.2011.27095
- Doane T, Horwath W (2003) Spectrophotometric determination of nitrate with a single reagent. *Anal. Lett.* 36:2713–2722
- Driebe E, Whitham T (2000) Cottonwood hybridization affects tannin and nitrogen content of leaf litter and alters decomposition. *Oecologia*. 123:99-107
- Earl DA, vonHoldt BM (2011) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour* 4:359–361. doi: 10.1007/s12686-011-9548-7
- Ehrenfeld JG (2003) Effects of exotic plant invasions on soil nutrient cycling processes. *Ecosystems* 6:503–523. doi: 10.1007/s10021-002-0151-3
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* 14:2611–20. doi: 10.1111/j.1365-294X.2005.02553.x
- Fox J, Weisberg S (2011) An (R) Companion to Applied Regression. Second Edition. Sage, Thousand Oaks, CA
- Goldstein CL, Williard KWJ, Schoonover JE, et al. (2006) Soil and groundwater nitrogen response to invasion by an

- exotic nitrogen-fixing shrub. *J Environ Qual* 39:1077–84. doi: 10.2134/jeq2009.0155
- Gu J, Yang M, Wang J, et al. (2010) Genetic diversity analysis of black locust (*Robinia pseudoacacia* L.) distributed in China based on allozyme markers approach. *Front Agric China* 4:366–374. doi: 10.1007/s11703-010-1023-9
- Hättenschwiler S, Vitousek P (2000) The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends Ecol Evol* 15:238–243
- Hierro J, Maron J, Callaway R (2005) A biogeographical approach to plant invasions: the importance of studying exotics in their introduced and native range. *J Ecol* 5–15. doi: 10.1111/j.1365-2745.2004.00953.x
- Hughes R, Denslow J (2005) Invasion by an N<sub>2</sub>-fixing tree alters function and structure in wet lowland forests of Hawaii. *Ecol Appl* 15:1615–1628
- Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23:1801–6. doi: 10.1093/bioinformatics/btm233
- Keane R, Crawley M (2002) Exotic plant invasions and the enemy release hypothesis. *Trends Ecol Evol* 17:164–170
- Le Roux JJ, Blignaut M, Gildenhuys E, et al. (2013) The molecular ecology of biological invasions: what do we know about non-additive genotypic effects and invasion success? *Biol Invasions* 16:997–1001. doi: 10.1007/s10530-013-0568-y
- LeBauer D, Treseder K (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89:371–379
- Lee CE (2002) Evolutionary genetics of invasive species. *Trends Ecol Evol* 17:386–391. doi: 10.1016/S0169-5347(02)02554-5
- Lee CR, Mitchell-Olds T (2011) Quantifying effects of environmental and geographical factors on patterns of genetic differentiation. *Mol Ecol* 20:4631–42. doi: 10.1111/j.1365-294X.2011.05310.x
- Lian C, Hogetsu T (2002) Development of microsatellite markers in black locust (*Robinia pseudoacacia*) using a dual-suppression-PCR technique. *Mol Ecol Notes* 211–213. doi: 10.1046/j.1471-8278
- Liesebach H, Schneck V (2012) Chloroplast DNA variation in planted and natural regenerated stands of black locust (*Robinia pseudoacacia* L.). *Silvae Genet* 61:27–35
- Liesebach H, Yang M, Schneck V (2004) Genetic diversity and differentiation in a black locust (*Robinia pseudoacacia* L.) progeny test. *For Genet* 11:151–161
- Madritch M, Hunter M (2002) Phenotypic diversity influences ecosystem functioning in an oak sandhills community. *Ecology* 83:2084–2090
- Madritch MD, Greene SL, Lindroth RL (2009) Genetic mosaics of ecosystem functioning across aspen-dominated landscapes. *Oecologia* 160:119–27. doi: 10.1007/s00442-009-1283-3
- Mazerolle MJ (2013) AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). In: R Package version 1.35. <http://cran.r-project.org/package=AICcmodavg>
- McKey D (1974) Adaptive patterns in alkaloid physiology. *Am Nat* 108:305–320
- McKey D (1979) The distribution of secondary compounds within plants. Herbivores: their interaction with secondary plant metabolites. Academic Press, New York (1979): 55-133
- Mishima K, Hirao T, Urango S, et al. (2009) Isolation and characterization of microsatellite markers from *Robinia pseudoacacia* L. *Mol Ecol Resour* 9:850–852. doi: 10.1111/j.1755-0998.2008.02302.x
- Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral pathogens. *Nature* 421:625–7. doi: 10.1038/nature01317
- Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. *Proc Natl Acad Sci* 98:5446–51. doi: 10.1073/pnas.091093398
- Mulvaney RL (1996) Extraction of exchangeable ammonium and nitrate and nitrite. *Methods soil Anal Part 3*:1129–1131
- Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R<sup>2</sup> from generalized linear mixed-effects models. *Methods Ecol Evol* 4:133–142. doi: 10.1111/j.2041-210x.2012.00261.x
- Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28:2537–9. doi: 10.1093/bioinformatics/bts460
- Pigliucci M (2005) Evolution of phenotypic plasticity: where are we going now? *Trends Ecol Evol* 20:481–6. doi: 10.1016/j.tree.2005.06.001
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155:945–59
- Röhm M, Werner D (1991) Nitrate levels affect the development of the black locust-*Rhizobium* symbiosis. *Trees* 227–231

- Sakai A, Allendorf F, Holt J (2001) The population biology of invasive species. *Annu Rev Ecol Syst* 305–332
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nat Biotechnol* 18:1–2
- Schweitzer JA, Bailey JK, Fischer DG, et al. (2008) Plant-soil microorganism interactions: heritable relationship between plant genotype and associated soil microorganisms. *Ecology* 89:773–81
- Simberloff D, Von Holle B (1999) Positive interactions of nonindigenous species: invasional meltdown? 21–32
- Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139:457–462
- Srivastava DS, Cadotte MW, MacDonald AM, et al. (2012) Phylogenetic diversity and the functioning of ecosystems. *Ecol Lett* 15:637–48. doi: 10.1111/j.1461-0248.2012.01795.x
- Surles S (1989) Allozyme variation in black locust (*Robinia pseudoacacia*). *Can J For* 19:471–479
- Surles S, Hamrick J, Bongarten B (1990) Mating systems in open-pollinated families of black locust (*Robinia pseudoacacia*). *Silvae Genet* 39:35–40.
- Suzuki S, Suzuki Y, Yamamoto N, et al. (2009) High-throughput determination of thioglycolic acid lignin from rice. *Plant Biotechnol* 26:337–340. doi: 10.5511/plantbiotechnology.26.337
- Treseder KK, Vitousek PM (2001) Potential ecosystem-level effects of genetic variation among populations of *Metrosideros polymorpha* from a soil fertility gradient in Hawaii. *Oecologia* 126:266–275. doi: 10.1007/s004420000523
- Vitousek PM (1990) Biological invasions and ecosystem processes: towards an integration of population biology and ecosystem studies. *Oikos* 57: 7-13
- Vitousek PM, Howarth R (1991) Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* 13:87–115
- Vitousek PM, Walker LR (1989) Biological invasion by *Myrica faya* in Hawai'i : plant demography, nitrogen fixation, ecosystem effects. *Ecol Monogr* 59:247–265
- Von Holle B, Joseph KA., Largay EF, Lohnes RG (2006) Facilitations between the introduced nitrogen-fixing tree, *Robinia pseudoacacia*, and nonnative plant species in the glacial outwash upland ecosystem of Cape Cod, MA. *Biodivers Conserv* 15:2197–2215. doi: 10.1007/s10531-004-6906-8
- Wiegner TN, Hughes F, Shizuma LM, et al. (2013) Impacts of an invasive N<sub>2</sub>-fixing tree on Hawaiian stream water quality. *Biotropica* 45:409–418
- Wright S (1943) Isolation by distance. *Genetics* 28:114–138.
- Yeates GW, Williams PA (2001) Influence of three invasive weeds and site factors on soil microfauna in New Zealand. *Pedobiologia* 45:367-383
- Yelenik SG, Stock WD, Richardson DM (2004) Ecosystem Level Impacts of Invasive *Acacia saligna* in the South African Fynbos. *Restor Ecol* 12:44–51. doi: 10.1111/j.1061-2971.2004.00289.x
- Yuan CQ, Li YF, Wang L, et al. (2013) Evidence for inbreeding depression in the tree *Robinia pseudoacacia* L. (Fabaceae). *Genet Mol Res* 12:6249–56. doi: 10.4238/2013.December.4.12
- Zheng Y, Harman D, Swartz H (2003) Resistance to locust leafminer (Coleoptera: Chrysomelidae) in black locust. *J Econ Entomol* 96:53–57

## Tables

**Table 1** Number of ramets 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** Frequency-based diversity indices 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 |
|------------------|---------|---------|---------|---------|---------|-----------------|
| <b>Native</b>    | 10.13   | 5.09    | 0.69    | 0.75    | 0.08    | 11              |
| <b>se</b>        | (1.33)  | (0.84)  | (0.04)  | (0.05)  | (0.04)  |                 |
| <b>Midwest</b>   | 9.88    | 4.98    | 0.71    | 0.77    | 0.07    | 4               |
| <b>se</b>        | (1.47)  | (0.70)  | (0.05)  | (0.04)  | (0.06)  |                 |
| <b>Northeast</b> | 9.25    | 4.94    | 0.68    | 0.77    | 0.11    | 8               |
| <b>se</b>        | (1.32)  | (0.67)  | (0.03)  | (0.04)  | (0.03)  |                 |
| <b>Mean</b>      | 9.750   | 5.002   | 0.692   | 0.760   | 0.086   | 7.7             |
| <b>se</b>        | (0.762) | (0.762) | (0.024) | (0.023) | (0.025) | (0.974)         |

**Table 3** ANOVA (Type III) results from linear mixed models with region as the fixed effect and genet as a random effect.  $R^2$  marginal ( $R^2_m$ ) and  $R^2$  conditional ( $R^2_c$ ) represent the amount of variation in leaf chemistry explained by the fixed effect and full model, respectively. The difference between marginal and conditional  $R^2$  ( $\Delta R^2$ ), reflects the variance explained by the random effect—genet identity.

| Leaf Chemistry | $\chi^2$ | df | p       | $R^2_m$ [95%CI]  | $R^2_c$ [95%CI]  | $\Delta R^2$ [95%CI] |
|----------------|----------|----|---------|------------------|------------------|----------------------|
| %N             | 71.27    | 2  | <0.0001 | 0.38 [0.25-0.49] | 0.66 [0.57-0.76] | 0.28 [0.18-0.40]     |
| %C             | 190.01   | 2  | <0.0001 | 0.62 [0.53-0.70] | 0.79 [0.73-0.85] | 0.17 [0.10-0.24]     |
| %Lignin        | 115.39   | 2  | <0.0001 | 0.39 [0.29-0.48] | 0.48 [0.37-0.59] | 0.09 [0.02-0.17]     |
| C:N            | 136.48   | 2  | <0.0001 | 0.56 [0.45-0.65] | 0.78 [0.73-0.85] | 0.22 [0.15-0.32]     |
| GAE (mg/g)     | 78.28    | 2  | <0.0001 | 0.43 [0.30-0.55] | 0.73 [0.66-0.82] | 0.30 [0.21-0.42]     |

**Table 4** ANOVA statistics and coefficients of determination for the best models determined by AICc.  $R^2$  marginal ( $R^2_m$ ) and  $R^2$  conditional ( $R^2_c$ ) represent the amount of variation in soil chemistry explained by the fixed effect and full model, respectively. The difference between marginal and conditional  $R^2$  ( $\Delta R^2$ ), reflects the variance explained by the random effect—genet identity.

| Chemistry        | Fixed Effect  | $\chi^2$ | p      | $R^2_m$ [95%CI]   | $R^2_c$ [95%CI]  | $\Delta R^2$ [95%CI] |
|------------------|---------------|----------|--------|-------------------|------------------|----------------------|
| $\Delta NH_4^+$  | Native Status | 4.22     | 0.040  | 0.04 [-0.04-0.09] | 0.44 [0.32-0.58] | 0.40 [0.29-0.56]     |
| $\Delta NO_3^-$  | Native Status | 3.08     | 0.080  | 0.03 [-0.04-0.08] | 0.46 [0.34-0.60] | 0.43 [0.32-0.59]     |
| $NH_4^+$         | Region        | 60.21    | <0.001 | 0.32 [0.19-0.43]  | 0.56 [0.45-0.67] | 0.24 [0.14-0.36]     |
| $NO_3^-$         | Region        | 14.54    | <0.001 | 0.13 [0.01-0.26]  | 0.60 [0.53-0.74] | 0.47 [0.37-0.63]     |
| Control $NH_4^+$ | Region        | 24.69    | <0.001 | 0.18 [0.05-0.29]  | 0.55 [0.44-0.68] | 0.37 [0.26-0.51]     |
| Control $NO_3^-$ | Region        | 92.94    | <0.001 | 0.20 [0.06-0.31]  | 0.59 [0.48-0.71] | 0.39 [0.28-0.54]     |

**Table 5** Wilcoxon rank sign test comparing regional averages of soil  $NO_3^-$  and  $NH_4^+$  from paired sites.

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

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**Table 6** Correlations (r) between leaf and soil chemistry for the a) Native, b) Midwest, and c) Northeast regions. Soil metrics are given in columns and leaf metrics are given in rows.

| Chemistry        | $\Delta \text{NH}_4^+$ | $\Delta \text{NO}_3^-$ | $\text{NH}_4^+$ | $\text{NO}_3^-$ | Control $\text{NH}_4^+$ | Control $\text{NO}_3^-$ |
|------------------|------------------------|------------------------|-----------------|-----------------|-------------------------|-------------------------|
| <b>Native</b>    |                        |                        |                 |                 |                         |                         |
| %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                   |
| <b>Midwest</b>   |                        |                        |                 |                 |                         |                         |
| %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                  |
| <b>Northeast</b> |                        |                        |                 |                 |                         |                         |
| %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:  $p < 0.001$  (\*\*\*),  $p < 0.01$  (\*\*),  $p < 0.05$  (\*)

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## FIGURE LEGEND

**Figure 1** Location of sampling sites. Each blue circle represents a sampling location. There are 30 sampling locations per region for a total of 90 sampling locations.

**Figure 2** Principle Coordinates Analysis of the three regions using *Cavalli-Sforza* chord distances.

**Figure 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 4** Descending average soil chemistry ( $\Delta$ ) 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 5** 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 1

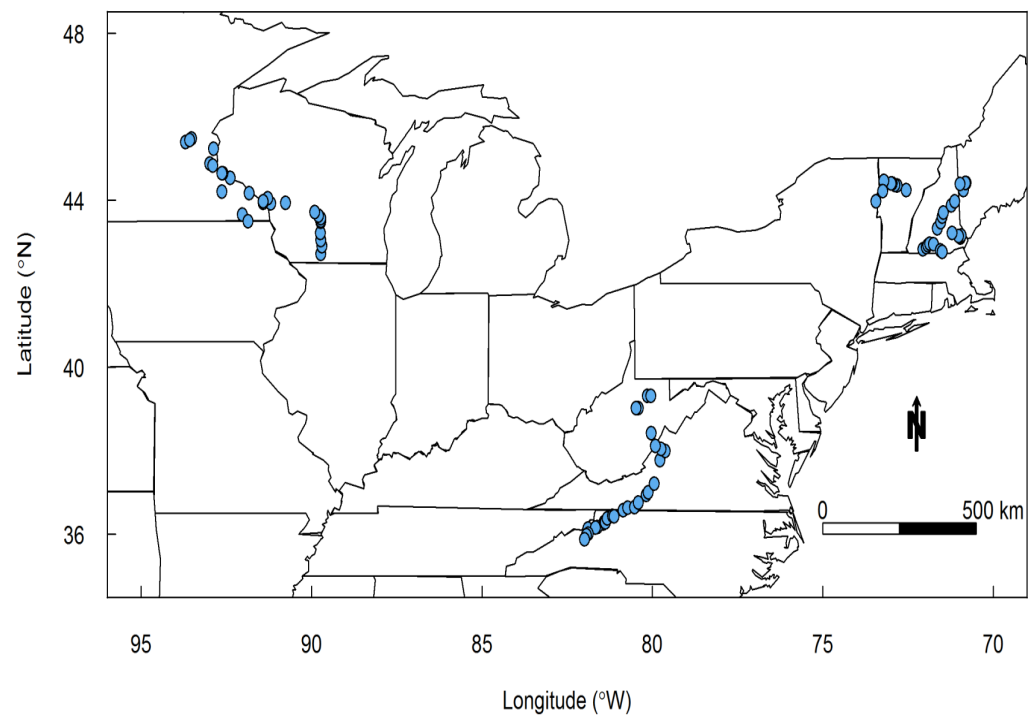


Figure 2

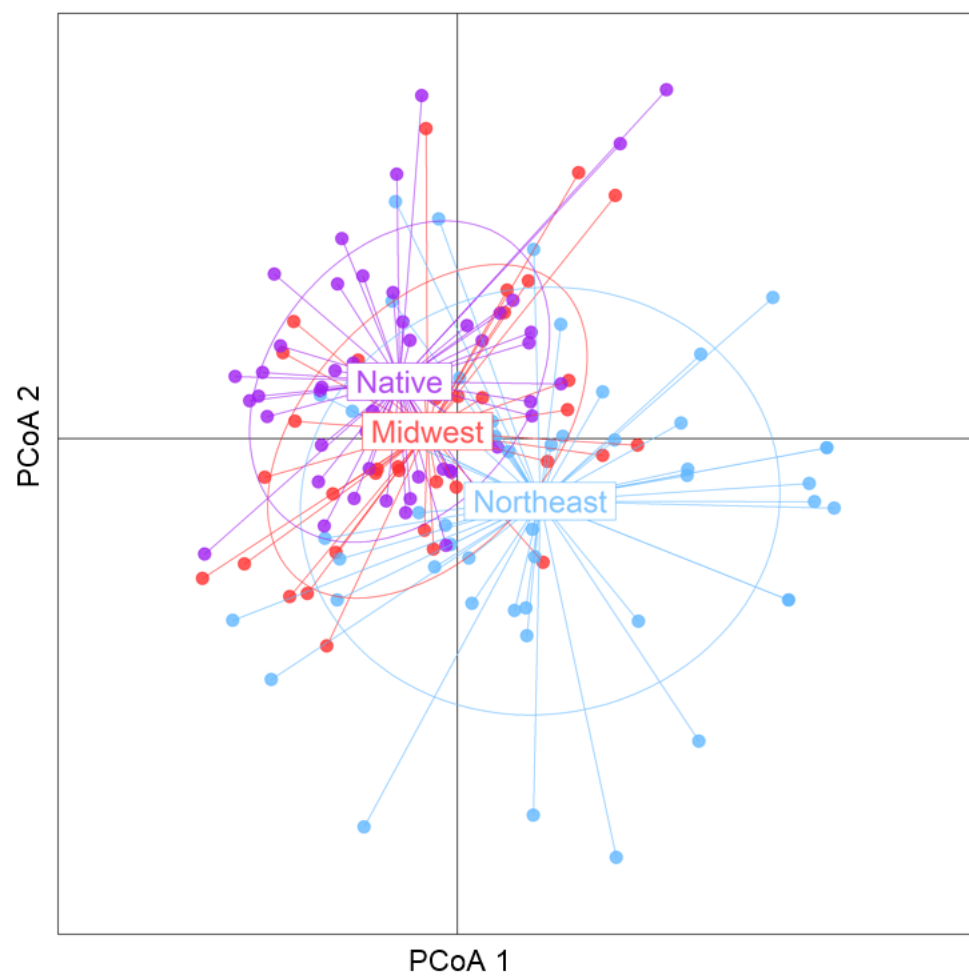


Figure 3

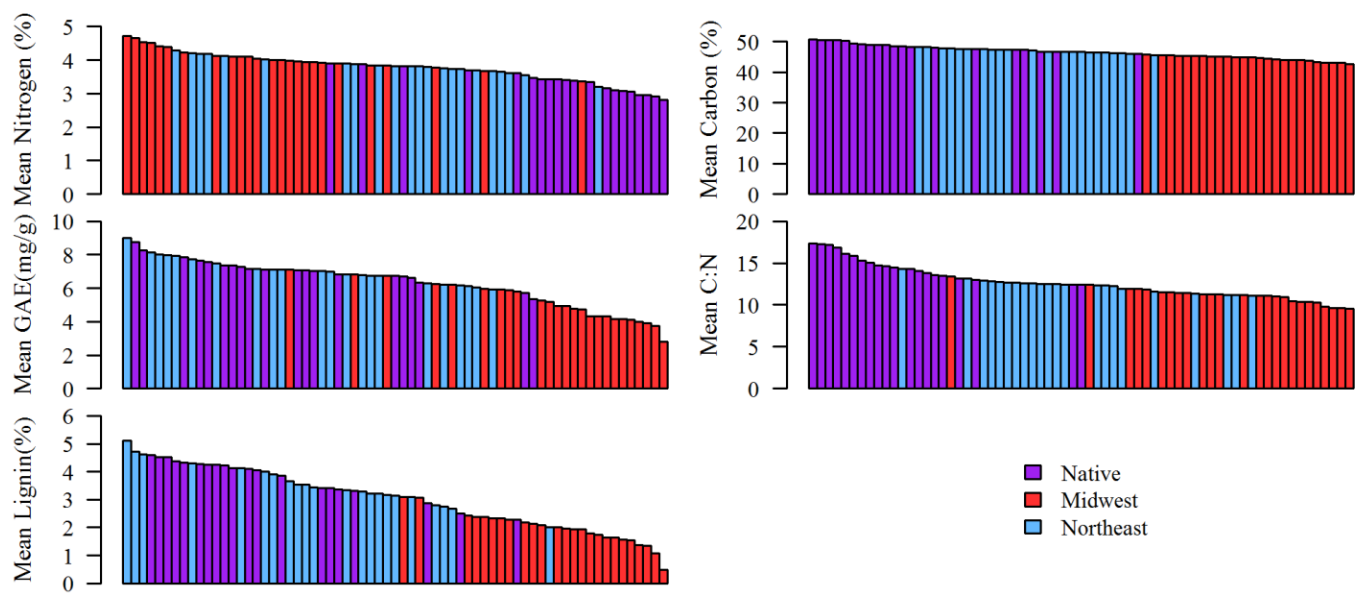


Figure 4

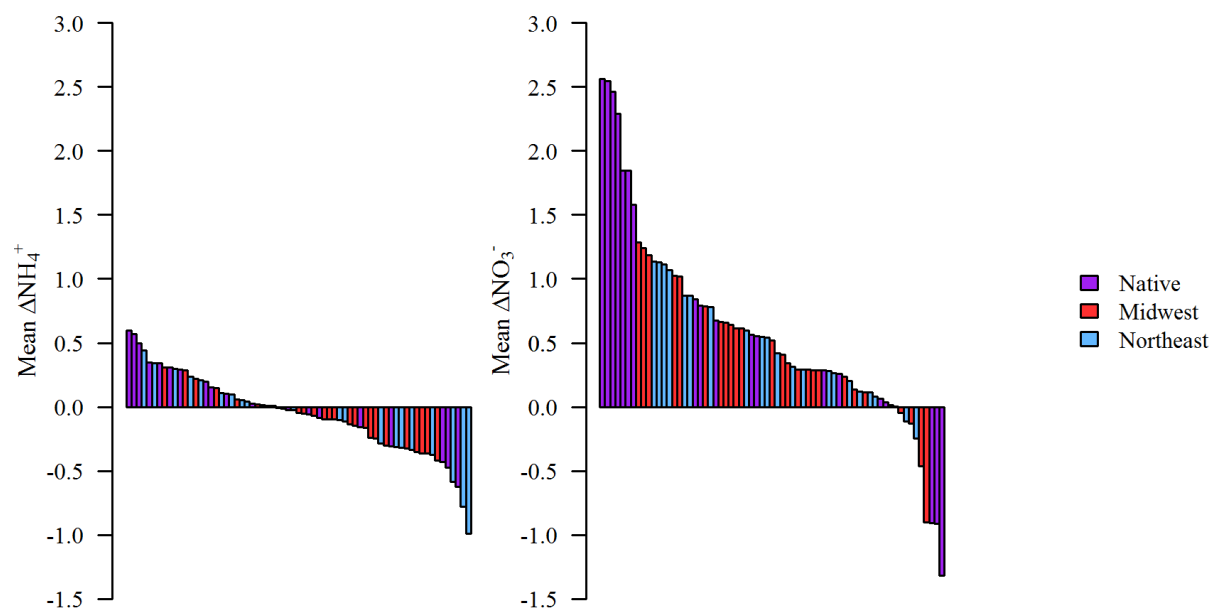


Figure 5

