



Symposium Article

Continent-Wide Climatic Variation Drives Local Adaptation in North American White Clover

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Abstract

Climate-associated clines in adaptive polymorphisms are commonly cited as evidence of local adaptation within species. However, the contribution of the clinally varying trait to overall fitness is often unknown. To address this question, we examined survival, vegetative growth, and reproductive output in a central US common garden experiment using 161 genotypes of white clover (*Trifolium repens* L.) originating from 15 locations across North America. White clover is polymorphic for cyanogenesis (hydrogen cyanide release upon tissue damage), a chemical defense against generalist herbivores, and climate-associated cyanogenesis clines have repeatedly evolved across the species range. Over a 12-month experiment, we observed striking correlations between the population of origin and plant performance in the common garden, with climatic distance from the common garden site predicting fitness more accurately than geographic distance. Assessments of herbivore leaf damage over the 2015 growing season indicated marginally lower herbivory on cyanogenic plants; however, this effect did not result in increased fitness in the common garden location. Linear mixed modeling suggested that while cyanogenesis variation had little predictive value for vegetative growth, it is as important as climatic variation for predicting reproductive output in the central United States. Together, our findings suggest that knowledge of climate similarity, as well as knowledge of locally favored adaptive traits, will help to inform transplantation strategies for restoration ecology and other conservation efforts in the face of climate change.

Subject area: Population structure and phylogeography

Key words: adaptive polymorphism, climatic adaptation, cline, common garden, cyanogenesis, environmental distance

Geographically widespread species can experience substantial environmental heterogeneity across their ranges, and populations frequently adapt to their local climates (Clausen et al. 1941; Hiesey et al. 1942; Kawecki and Ebert 2004). Local adaptation is thought to be common across all domains of life (Leimu and Fischer 2008; Hereford 2009). In the face of rapid climate change, the ability to adapt to local climate may be particularly important for plant species due to their sessile nature (Alberto et al. 2013). Studies of local adaptation in plants are therefore informative for identifying species

that can readily adapt to local climate, and for quantifying the relative importance of specific traits for local adaptation. Insights into local climatic adaptation also provide useful information for conservation and restoration efforts.

Home-site fitness advantage (i.e., higher fitness for the local genotype in its local environment) is central to the concept of local adaptation and has been demonstrated in plant species for many years through common garden experiments (Clausen et al. 1940; Leimu and Fischer 2008). Kawecki and Ebert (2004) suggested that

geographic distance and/or ecological distance, defined by quantitative environmental parameters, may be used as explanatory variables for fitness variation in common garden experiments. Previous studies comparing these 2 distance measures have suggested that geographic distance can be a good predictor of local adaptation at broad spatial scales (>200 km) (Galloway and Fenster 2000; Becker et al. 2006). In contrast, environmental distance has been found to be a better predictor of success in ecological restoration experiments (Montalvo and Ellstrand 2000; Raabová et al. 2007). More generally, different contributors to total fitness, including survival, vegetative growth, and flowering phenology, have been found to show a variety of responses to climatic factors across species ranges (Olsson and Ågren 2002; Prieto et al. 2008; Haggerty and Galloway 2011; Samis et al. 2012; Moles et al. 2014; Preite et al. 2015; Siepielski et al. 2017).

Many studies of local adaptation have focused on polymorphic traits with simple genetic underpinnings and identifiable locally adaptive functions (e.g., Colosimo et al. 2005; Kivimäki et al. 2007; Linnen et al. 2013; Savolainen et al. 2013; Tiffin and Ross-Ibarra 2014). Due to the challenges of directly measuring fitness in natural settings, the importance of these traits for overall fitness and local adaptation is commonly inferred indirectly, either from observations of correlations between environmental gradients and genotype (or phenotype) frequencies (e.g., Baxter et al. 2010), or from short-term experiments in controlled conditions that may not generalize to natural settings (Anderson et al. 2011, Jacobs and Latimer 2012). Fewer studies have directly assessed the fitness impact of polymorphic traits in field experiments (e.g., Hall and Willis 2006; Wadgyamar et al. 2017). In an attempt to add to our understanding of the process of local adaptation, this study uses fitness measures from an experimental field plot to assess the ability of a well-documented, locally adaptive chemical defense polymorphism to predict overall fitness variation. The relative predictive values of geographic versus environmental distance (specifically, climatic distance) for local adaptation are also explicitly examined.

The White Clover Cyanogenesis Polymorphism

Cyanogenesis, the production of hydrogen cyanide upon tissue damage, occurs in >3000 species across the plant kingdom (Gleadow and Møller 2014). It is generally accepted to have evolved as a chemical defense against generalist herbivores. While this trait is typically universally present in individuals of cyanogenic species, white clover (*Trifolium repens* L.) is unusual in that both cyanogenic and acyanogenic plants can be found within populations (Armstrong et al. 1913). The cyanogenesis polymorphism is manifested geographically as climate-associated clines where the frequency of cyanogenic plants decreases with increasing latitude and altitude; thus, higher proportions of cyanogenic plants are found in warmer climates. Cyanogenesis clines have evolved in both the native European species range as well as in introduced white clover populations worldwide (Daday 1954a, 1954b, 1958; De Araújo 1976; Till-Bottraud et al. 1988; Caradus et al. 1990; Kooyers and Olsen 2012, 2013; Kooyers et al. 2014; Thompson et al. 2016). These patterns of repeated cline evolution provide evidence for strong selection on the cyanogenesis polymorphism and for local climatic adaptation that is specifically related to this phenotype. Proposed selective factors for cyanogenesis cline evolution include climatically varying herbivore abundance, fitness costs of cyanogenesis in colder climates, and potential benefits of cyanogenic components for functions other than herbivore deterrence (e.g., cyanogenic glucosides may serve a function in drought

stress adaptation) (Hughes 1991; Kooyers and Olsen 2012, 2013; Kooyers et al. 2014; Thompson et al. 2016).

At the biochemical level, cyanogenesis in white clover results from the interaction of 2 components that are spatially separated in intact tissue, cyanogenic glucosides and their hydrolyzing enzyme, linamarase (Gleadow and Møller 2014). Two unlinked Mendelian genetic polymorphisms control the presence/absence of the 2 components (*Ac/ac* and *Li/li* for cyanogenic glucosides and linamarase, respectively); the dominant allele of each gene confers the presence of the component, and homozygous recessive genotypes lack the component (Hughes 1991; Olsen et al. 2013). At the molecular level, both *Ac/ac* and *Li/li* are gene presence/absence polymorphisms, with recessive alleles corresponding to recurrently-evolved gene deletions (Olsen et al. 2007, 2008, 2013). Thus, 4 “cyanotypes” are present in white clover populations. Cyanogenic plants (AcLi) produce both components, whereas acyanogenic plants (AcLi, acLi, and acLi) lack one or both components and do not produce HCN.

In this study, we performed a white clover common garden experiment in a central US location to assess local adaptation across North American white clover populations. We used 161 wild genotypes sampled from 15 geographically widespread locations to examine fitness variation as it relates to population of origin and cyanogenesis variation. While the use of a single common garden site does not allow for documentation of reciprocal home-site advantage (see Discussion), it can nonetheless reveal fitness variation as related to the population of origin (Rutter and Fenster 2007; Preite et al. 2015; Peterson et al. 2016). We asked the following specific questions: 1) To what extent do geographic and/or climatic distance predict fitness variation across populations? 2) Do cyanogenic plants experience less herbivore leaf damage than acyanogenic plants, resulting in higher fitness regardless of the population of origin? And 3) Which combinations of climate parameters and cyanotype best predict growth and fecundity variation in the common garden location?

Materials and Methods

Study System

Trifolium repens is a perennial allotetraploid herbaceous legume that is obligately outcrossing and primarily bee pollinated. In addition to reproduction by seed, it spreads vegetatively by stolons, allowing for the study of multiple clonal replicates per genotype in field experiments. White clover was an important source of soil nitrogen for agriculture before the advent of synthetic fertilizers and was therefore intentionally introduced across temperate and cool tropical regions worldwide with European colonization (Kjærsgaard 2003); it remains an important temperate forage crop. Due to its history of repeated, intentional introductions, non-native populations contain extensive standing genetic variation that natural selection has acted upon (Kooyers and Olsen 2014). White clover has extremely large effective population sizes worldwide and displays minimal population structure on continental and global scales (George et al. 2006; Olsen et al. 2007; Kooyers and Olsen 2012, 2013).

Sampling

Plant samples were collected from 15 North American populations ranging from central Florida to Vancouver, British Columbia during the 2014 growing season (Figure 1, Table 1, Supplementary Table S1). Samples were collected as stolon cuttings (STL and GFL populations) or mature seeds (all other populations) from 9 to 11 plants per

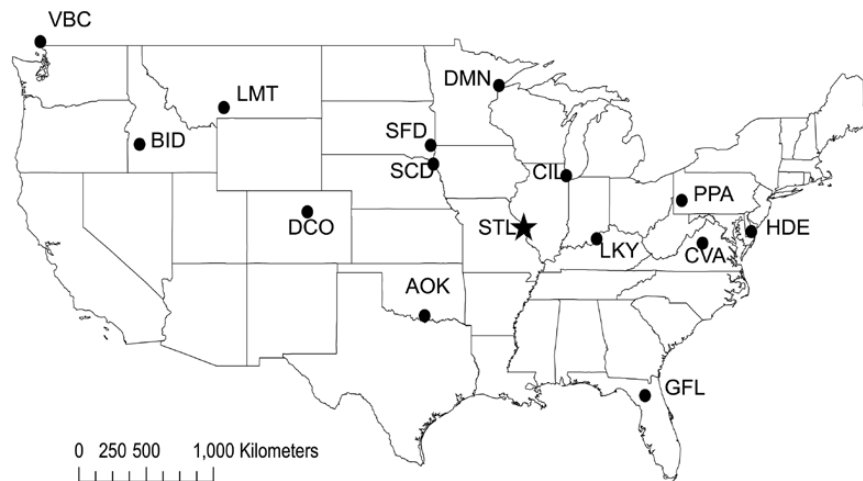


Figure 1. Sampling locations and abbreviations for the 15 populations used in the common garden experiment. The star indicates the location of the common garden experiment in St. Louis, MO (STL).

Table 1. Geographic coordinates and distances to the common garden site for the 15 populations used in this study

Population ^a	ID	N	Latitude	Longitude	Distance (km)	Relative distance
Ardmore, OK	AOK	48	34.16	-97.14	789.1	0.28
Boise, ID	BID	11	43.60	-116.22	2232.0	0.79
Chicago, IL	CIL	9	41.96	-87.63	433.1	0.15
Charlottesville, VA	CVA	11	38.01	-78.52	1028.0	0.36
Denver, CO	DCO	11	39.78	-104.97	1270.0	0.45
Duluth, MN	DMN	11	46.79	-92.15	919.7	0.32
Gainesville, FL	GFL	32	29.64	-82.36	1237.0	0.44
Harbeson, DE	HDE	10	38.73	-75.28	1301.0	0.46
Louisville, KY	LKY	10	38.28	-85.62	408.3	0.14
Livingston, MT	LMT	11	45.65	-110.56	1838.0	0.65
Pittsburgh, PA	PPA	11	40.45	-79.95	908.8	0.32
Sioux City, SD	SCD	11	42.54	-96.53	682.4	0.24
Sioux Falls, SD	SFD	11	43.51	-96.73	764.7	0.27
St. Louis, MO	STL	32	38.64	-90.29	0	0.00
Vancouver, BC	VBC	11	49.22	-122.82	2830.0	1.00

^aContributions to sample collections are acknowledged in Supplementary Table S1. Collection dates are also listed.

population, for a total of 161 unique genotypes that were used in a common garden experiment near St. Louis, MO. Collections were spaced a minimum of 5 m apart to prevent sampling multiple ramets or seed heads from the same genet; GPS coordinates were recorded for each sample. To control for potential confounding effects of sampling stolon cuttings (which represent a subset of genotypes that survived to maturity in their local climate) rather than seeds at the STL and GFL sites, analyses of local adaptation were performed both with and without those populations. For 3 of the populations (AOK, GFL, and STL), deeper sampling was performed largely from stolon cuttings to assess neutral genetic differentiation among populations (32–48 samples per population; Supplementary Tables S1 and S2). To calculate the geographic distance between the common garden site and sampled populations, latitudes and longitudes were averaged across samples within each population, and the great circle distance was calculated using the haversine formula (Veness 2012; Table 1, Supplementary Table S2).

All samples were grown in the Washington University (WU) greenhouse in 4" round pots filled with MetroMix 360 soil before genetic analyses and the field experiment (Supplementary Table S1). Rooting hormone was applied to plants collected as stolon cuttings

to encourage establishment on mist benches. For samples collected as seed, 10 seeds per maternal parent were scarified using fine grit sandpaper and planted in a single pot on mist benches. Upon germination, one seedling was randomly selected for use in further analyses, and others were discarded. We were not able to reduce potential maternal effects by producing a second generation of seed in the greenhouse, as self-incompatibility in white clover makes this impractical when using wild population sample collections.

Population Structure Analyses

For the 3 intensively sampled populations (AOK, GFL, and STL), genomic DNA was extracted for genotyping-by-sequencing (GBS) using a DNA extraction protocol modified from Whitlock (2008) with 120–150 mg young leaf tissue (Elshire et al. 2011; Supplementary Table S2). Leaf tissue for each sample was ground in liquid nitrogen using mortars and pestles. Columns from the *IBI Scientific* Genomic DNA Mini Kit (Plant) were used for filtration and binding steps.

DNA samples were submitted to Cornell University's Institute for Genomic Diversity for library preparation and GBS using the Illumina HiSeq 2000 platform. Quality control and SNP calling were

performed on raw GBS data by Cornell using the UNEAK pipeline and TASSEL v3.0.166 (Lu et al. 2013). UNEAK was developed for polyploid species that lack reference genomes and provides a stringent filtering system to account for highly repetitive sequences. Read depth was calculated with VCFtools v0.1.11 (Danecek et al. 2011). SNPs were called using a minor allele frequency cutoff of 0.01.

To assess genetic differentiation between the AOK, GFL, and STL populations, pairwise F_{ST} values were calculated using the filtered SNP data set in GenAlEx 6.5 (Peakall and Smouse 2006). For a comparison to this background genomic F_{ST} , pairwise F_{ST} was also calculated separately for the *Ac* and *Li* cyanogenesis genes using genotypes inferred from cyanogenesis phenotyping and genotyping. *Ac* and *Li* allele frequencies were calculated with the Hardy–Weinberg assumption that the frequency of homozygous recessive genotypes is equal to q^2 within each population (Kooyers and Olsen 2012).

Cyanogenesis Phenotyping and Genotyping

For each genotype, phenotyping for the presence/absence of HCN production in leaf tissue was performed using Feigl–Anger tests, as described previously (Olsen et al. 2007). For acyanogenic individuals, the presence/absence of each cyanogenic component (i.e., cyanogenic glucosides or linamarase) was determined by exogenous addition of the complementary component. Negative reaction results were repeated at least twice to minimize false negatives. To confirm that cyanogenesis phenotyping results corresponded to *Ac* and *Li* gene presence/absence, DNA was extracted with the Genomic DNA Mini Kit (Plant) kits (IBI Scientific) using 100 mg young leaf tissue, and PCR was performed for the *Ac* and *Li* loci using previously described primers (Olsen et al. 2007, 2008). The presence of a PCR product was taken as evidence of gene presence. Negative results were confirmed by repeating the reaction at least twice. Fewer than 3% of PCR assays did not match phenotyping results.

Common Garden Establishment

Three replicate cuttings were made from each of the 161 unique genotypes, for a total of 483 plants (Supplementary Table S3). Care was taken to establish cuttings of the same size, including similar root masses and numbers of leaves. Rooting hormone was applied to encourage establishment. Cuttings were grown on mist benches in the WU greenhouse for 1 week and then were allowed to become established for an additional week under standard greenhouse conditions before being planted in the field.

The common garden experiment was conducted from April 2015 through March 2016. Established cuttings were transplanted to an experimental research garden plot at the WU Tyson Research Center in Eureka, MO (Supplementary Figure S1). The experimental plot was enclosed by an underground concrete barrier to exclude burrowing rodents and by a fence to exclude deer and other large mammals. The soil substrate consisted of local, native prairie soil. Planting occurred on 11 April 2015 to coincide with the spring leaf flush of local clover populations. Replicate cuttings were planted in a blocked design to account for environmental heterogeneity across the plot; one replicate per genotype was planted in a randomized design within each of 3 replicate blocks. Cuttings were watered only upon transplantation, after which they were left exposed to local environmental conditions for the remainder of the 12-month experiment.

All data collection in the field plot was performed blind with respect to the cyanotype and population origin of each plant. To

prevent intermingling of genotypes that would lead to inaccurate fitness measurements, plants were trimmed to 12 × 12-inch squares, with 6-inch gaps on all sides (Supplementary Figure S1). Trimming was performed by hand using scissors at 2- to 6-week intervals, depending on the rate of growth; plants were trimmed 8 times in 2015. Weeds were also removed from the plot to allow for accurate fitness measurements. As white clover generally performs best in areas with regular grazing or mowing (Andrae 2016), this trimming and weeding regime is not suspected to have unduly biased fitness measures.

Common Garden Fitness Measurements

Growth and Survival

Vegetative growth and tissue survival were assessed using digital photographs of each plant taken at 4-time points: 30 April; 24 May (before first trim); 18 October (following last trim); and 23 March 2016. Photos were taken directly over each plant, using a red-painted penny for color contrast and scale. *Easy Leaf Area* software (Easlon and Bloom 2014) was used to quantify total vegetative tissue surface area (Figure 2a). All output photos with highlighted quantified pixels were visually checked for quality. From these data, relative growth for the growing season was calculated as the difference in vegetative tissue area from April to October divided by the largest difference. In addition, biomass was collected with the first trim to verify that that vegetative area can serve as an accurate proxy for biomass production (Supplementary Methods).

Fecundity

White clover inflorescences are composed of tens to hundreds of individual florets, each capable of producing 1–8 seeds. Therefore, inflorescence count was used to measure fecundity because it was found to be significantly correlated with both seed mass and dried floral mass (Supplementary Methods; Supplementary Figure S2) (see also Kooyers et al. 2014 and references therein for similar measures of white clover reproductive output). Inflorescences were counted and removed from each plant once the oldest (basal) florets began turning downward, an indication of successful pollination.

Herbivory

Herbivore leaf damage was assessed 4 times (29 May, 2 July, 18 July, and 5 August) using a modified protocol of Kooyers et al. (2014) (Dirzo and Harper 1982a, 1982b), in which leaf tissue damage was quantified in an ordinal fashion as 0%, 1–25%, 26–50%, 51–75%, or >75% for all leaves on a randomly chosen stolon (Supplementary Figure S3). Data across the 4 sampling points were combined, and 2 herbivory metrics were calculated (Supplementary Table S3). Total herbivore leaf damage was calculated as the number of leaves with any herbivore damage, regardless of damage category, divided by the total number of leaves. Weighted herbivore leaf damage was calculated as the sum of leaf damage categories (A = 0, B = 0.25, C = 0.5, D = 0.75, E = 1), each multiplied by the number of leaves in their respective category.

Germination Experiment

Because we used clonally replicated cuttings of greenhouse-grown plants in the common garden experiment, fitness measures for these plants do not capture selection that occurs at early life stages, when germinants might be particularly susceptible to mortality from herbivore damage. Therefore, to address whether germinant fitness in the field is affected by cyanotype variation, we performed a germination

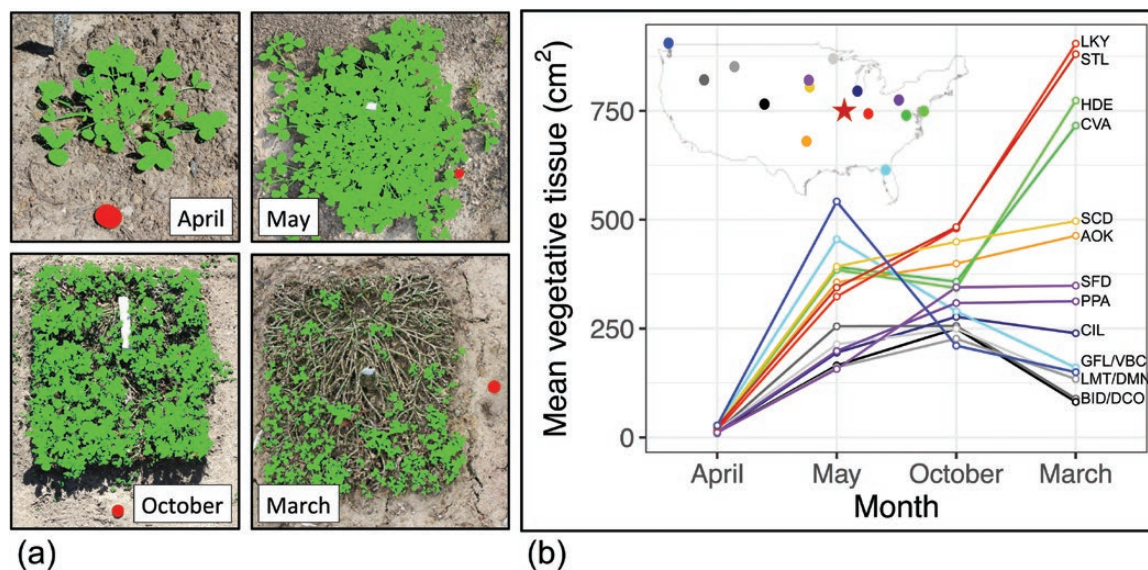


Figure 2. *Easy Leaf Area* output photos (a) are shown for a single plant at each photographic time point in the common garden experiment. Vegetative tissue is highlighted in green pixels, with a red-painted penny used for scale. The line graph (b) displays mean vegetative tissue across populations at each of the 4 time points. Lines that are similar in color represent populations that experienced similar trajectories over all 4 time points.

experiment at the common garden site using seeds that originated from the same maternal parents as the common garden genotypes (Supplementary Methods). We compared the cyanotype frequencies of the common garden genotypes (germinated in the greenhouse) to the germinants that survived to the seedling stage at the common garden site using a chi-squared contingency test.

Climate Principal Components Analysis and Distance Calculations

To quantify home-site climate variation across the 15 populations used in this study, we downloaded 19 bioclimatic variables related to temperature and precipitation (BIOCLIM, Hijmans et al. 2005), as well as annual potential evapotranspiration data (CGIAR; Trabucco and Zomer 2009), using averaged latitudes and longitudes for each population (Table 1). To evaluate the relationship between home-site climate and fitness performance specifically during the growing season, when most fitness data were collected, we removed 3 variables related exclusively to winter months (Bio 6 = min temperature of coldest month, Bio 11 = mean temperature of coldest quarter, and Bio 19 = precipitation of coldest quarter). To reduce multicollinearity among climatic variables (Farrar 1967), we performed a principal components analysis (PCA) using the *princomp()* function in R and utilized the top 3 PCs for subsequent analyses (R Core Team 2015). We calculated climatic distances between each population and the St. Louis common garden site for each PC as the Euclidean distances between the PC score of STL and each of the 14 “away” populations, generating *PC1_euc*, *PC2_euc*, and *PC3_euc* parameters. We then calculated an overall climate PC index as the sum of the 3 *PC_euc* values for each population. In these metrics, lower values indicate climates that are more similar to St. Louis.

Statistical Analyses and Linear Modeling

All statistical analyses were performed using R statistical software (v. 3.3.0, R Core Team 2015). Figures were generated with the *ggplot2* package (Wickham 2009). The *reshape2* and *plyr* packages

were used to format and summarize data for plots (Wickham 2007, 2011). For all fitness measures, we averaged data across the 3 replicate cuttings for each genotype and used this dataset of 161 averaged genotypes for subsequent statistical analyses.

To determine whether geographic distance or climatic distance is a better predictor of plant fitness in the St. Louis common garden site, we tested for correlations between distance measures (relative geographic distance to St. Louis and climate PC index) and key fitness measures (relative growth in vegetative tissue and inflorescence count). For these 4 comparisons, we calculated mean fitness measures for each population and performed Pearson correlation tests using the resulting 15 data points. The analysis was also performed excluding the 2 locations where stolon cuttings rather than seeds were sampled. We then created linear models using the *lm()* function in R and utilized adjusted R^2 values of the lines of best fit to compare the predictive abilities of geographic and climatic distances.

Using the 2 herbivory metrics, we performed pairwise Wilcoxon signed-rank tests between cyanogenic plants (AcLi) and each of the 3 acyanogenic groups to test for preferential feeding on acyanogenic plants. If preferential feeding on acyanogenic plants were associated with reduced fitness, we would expect the cyanogenic group to have elevated growth or reproduction relative to the acyanogenic groups. We, therefore, compared fitness measures (relative growth and inflorescence count) of the cyanogenic and acyanogenic groups using additional pairwise Wilcoxon signed-rank tests.

To compare the abilities of different combinations of climate parameters and cyanotype to predict fitness variation in the common garden location, we built sets of linear mixed models separately for 2 fitness response variables (relative growth in vegetative tissue and inflorescence count) using all combinations of *PC1_euc*, *PC2_euc*, *PC3_euc*, and cyanotype as parameters. We then performed multimodel inference and model averaging and calculated parameter weights across models to identify the most relevant parameters for predicting each fitness measure (Botero et al. 2014; see Burnham and Anderson 2002). Details on model construction and averaging are presented in the Supplementary Methods.

Results

Population Structure and Cyanogenesis Variation across Sampled Populations

Genotyping by sequencing (GBS) was performed for 112 individuals from 3 of the sampled locations (AOK, GFL, and STL) to assess neutral population differentiation across the sampled species range. Due to the high stringency of the UNEAK pipeline, which was designed for GBS data in polyploid species lacking reference genomes (Lu et al. 2013), the raw data (>2 million Illumina sequence reads) were filtered to 62 372 reciprocal sequence pairs for SNP calling. The average read depth per site was 3.37 \times . From these filtered sequences, 843 bi-allelic SNPs were identified and utilized for pairwise population F_{ST} calculations. We found negligible population structure, with all pairwise F_{ST} values <0.03 (Supplementary Table S4). These results corroborate previous findings that white clover shows very little population structure on regional and continental scales (George et al. 2006; Olsen et al. 2007; Kooyers and Olsen 2012, 2013).

Cyanotype frequencies varied widely among the 15 sampled populations, with the frequency of cyanogenic (AcLi) plants broadly corresponding to latitude and minimum winter temperature as in previously documented cyanogenesis clines (e.g., Kooyers and Olsen 2012, 2013) (Supplementary Figure S4). Consistent with this pattern, pairwise F_{ST} values for the *Ac* and *Li* cyanogenesis loci, which are expected to be under selection in cyanogenesis clines, were elevated by up to an order of magnitude between climatically distinct population pairs relative to the background genomic F_{ST} (Supplementary Table S4).

Fitness Variation

Growth and Survival

Supplementary Table S5 provides summary statistics for survivorship and total vegetative tissue area (cm²) of the 483 common garden plants (triplicate clones of 161 genotypes) at 4-time points: April, May, October, and March. Mortality was very low throughout the experiment. Three plants from different source populations (CVA, PPA, BID) had died by the end of the growing season in October. From October to March, 24 additional plants died, with mortality overrepresented in a subset of the populations (DCO = 4, DMN = 4, GFL = 4, LMT = 3, and VBC = 4), all of which are geographically distant and climatically distinct from the common garden site.

All populations increased in average vegetative tissue area during the establishment period from April to May (Figure 2b). However, populations varied widely in their vegetative growth from May to October, with some showing increased vegetative tissue area and others showing static or decreased tissue area. At the end of the growing season (October), the local St. Louis (STL) population had the highest mean vegetative tissue area remaining, followed closely by the geographically proximal Louisville population (LKY). Overall, the relative growth of plants in the common garden displayed a clear correlation with source population distance. Populations located closer to St. Louis had higher relative growth than those collected from more distant sites ($R^2 = 0.54$, $P = 0.001$; Figure 3a). This pattern remained significant when population samples that were collected as stolon cuttings (STL, GFL) were excluded from the analysis ($R^2 = 0.45$, $P = 0.007$; Supplementary Figure 5a). Thus, relative growth based on vegetative area indicated a home-site

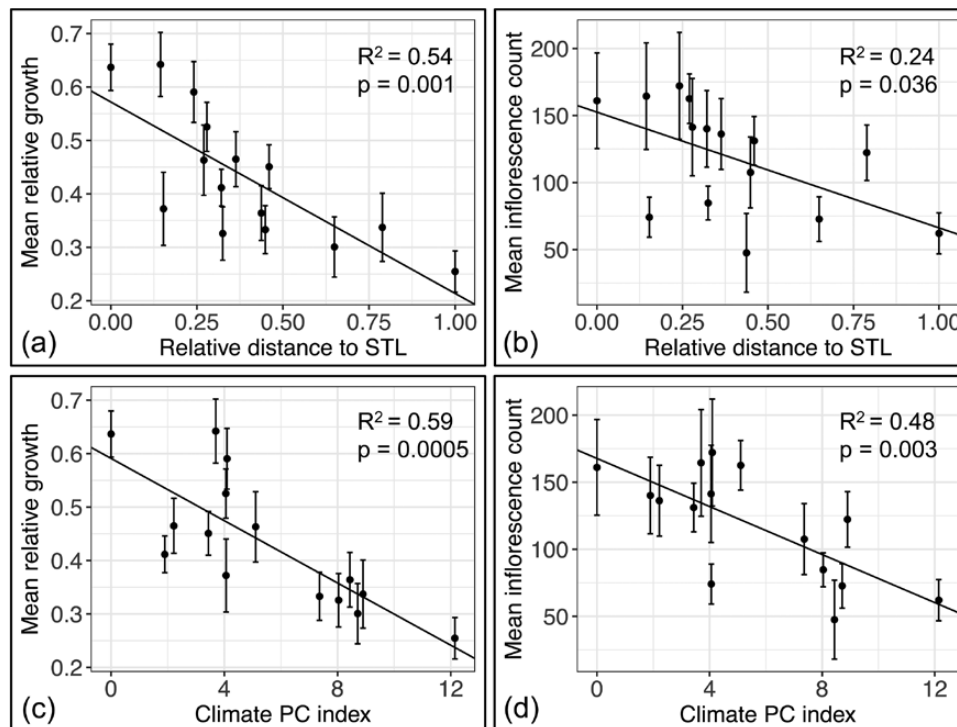


Figure 3. Linear relationships of 2 fitness measures (relative growth in vegetative tissue from April to October (a, c), and inflorescence count (b, d)) as a function of geographic distance (a, b) or climatic distance (PC index; c, d) across 15 populations. Data points show the mean value for each population with standard error bars. Adjusted R^2 values and P -values for lines of best fit are shown.

fitness advantage among white clover populations, with a gradation in fitness as a function of geographic distance from the source population to the common garden location.

As with vegetative growth during the growing season, changes in average vegetative tissue area over the winter (October to March) varied widely among populations in a pattern consistent with local adaptation. The 2 populations with the highest mean vegetative tissue area in both October and March were the same 2 populations that displayed the greatest relative growth during the main growing season: STL and LKY (red lines, Figure 2b). Two east coast US populations from similar latitudes to St. Louis (HDE and CVA) also performed well from October to March, despite the fact that they declined over the summer months (green lines).

Fecundity

Over the course of the growing season (April through October), the 483 plants produced 57385 inflorescences, and the average floral production was 119 inflorescences. Fifteen plants produced no flowers, 11 of which originated from 5 genotypes of the southernmost population (GFL). Additional summary statistics are presented in Supplementary Table S5. Total inflorescence count was positively correlated with relative growth in vegetative tissue. This held true both at the level of genotype ($R^2 = 0.06$, $P = 0.0009$) and population ($R^2 = 0.66$, $P = 0.0001$) (Supplementary Figure S2c,d).

Similar to relative growth in vegetative tissue, mean inflorescence production was correlated with distance of the source population from the experimental plot, with populations originating from sites nearer to St. Louis producing more inflorescences on average than those from more distant locations ($R^2 = 0.24$, $P = 0.036$; Figure 3b); this correlation remained marginally significant when the STL and GFL populations were removed ($R^2 = 0.23$, $P = 0.056$; Supplementary Figure 5b). The SCD, LKY, SFD, and STL populations, all from the central United States, had the highest mean inflorescence counts.

Floral production varied over the growing season, increasing in June and decreasing in September for all populations (Supplementary Figure S6). Plants displayed collective bursts of flowering following rainfall events (Supplementary Figure S7). The rate and magnitude of this flowering response varied across populations, with populations that produced the highest inflorescence counts over the season responding most strongly during flowering bursts (red lines, Supplementary Figures S6 and S7).

Herbivory

Total leaf damage was low overall compared to recent studies in white clover (e.g., Kooyers et al. 2014; Thompson and Johnson 2016), with only 10–12% of leaves showing any sign of leaf herbivore damage and no clear patterns across populations (Supplementary Figure S8a). Nonetheless, despite low herbivore leaf damage in the St. Louis common garden location, pairwise comparisons between cyanotypes revealed a nonsignificant trend, with cyanogenic (AcLi) plants showing less total herbivore leaf damage than all 3 classes of acyanogenic plants (Figure 4). Weighted herbivore leaf damage revealed the same trend (Supplementary Figure S8b).

Although cyanogenic (AcLi) plants showed a trend toward lower herbivore leaf damage, this did not translate into increased fitness (Supplementary Figure S8c,d). Rather, the cyanotype with both the highest relative growth and inflorescence count was AcLi (cyanogenic glucosides present but linamarase absent). While these trends in increased fitness for AcLi were not statistically significant, it bears noting that this cyanotype is the most common cyanotype in local

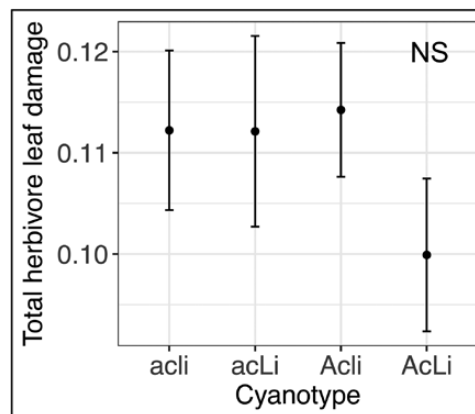


Figure 4. Variation in total herbivore leaf damage across cyanotypes. Mean values are shown for each cyanotype with standard error bars. Pairwise Wilcoxon signed-rank tests between the cyanogenic group (AcLi) and each of the 3 acyanogenic groups were not significant at the $P < 0.05$ level.

populations in the STL region (Supplementary Figure S4; Kooyers and Olsen 2012). These results provide the first empirical evidence that the most common local cyanotype shows marginally higher fitness than the other cyanotypes in the local climate.

Germination Experiment

Cyanotypes of surviving germinants in the common garden plot and greenhouse are presented in Supplementary Table S6. There was no significant difference in cyanotype proportions under the 2 growing conditions ($\chi^2 = 0.71$, $P = 0.87$). This result suggests that cyanotype does not affect fitness at early life stages, at least in the central US location and year of this study. Therefore, fitness measurements made from the clonal replicates in the common garden are apparently not missing a key component of cyanogenesis-related fitness variation at the germinant life stage.

Climate PCAs and Distance Calculations

In a PCA utilizing 16 Bioclim variables and annual potential evapotranspiration (Apet) data, PC1 explained 44% of the variance in climate among the 15 populations studied (Table 2). PC1 is driven primarily by variables related to precipitation (e.g., annual precipitation, precipitation in the driest month and quarter, but also annual mean temperature) (Supplementary Figure S9a). PC2 explained 24% of the variance in climate and is driven primarily by variables related to maximum and mean summer temperatures, as well as Apet (Supplementary Figure S9b). Lastly, PC3 explained 16% of the variance and corresponds to yearly temperature variability (e.g., isothermality, temperature seasonality) (O'Donnell and Ignizio 2012) (Supplementary Figure S9c). Climatic distances, calculated as Euclidean distances between PC scores of STL and the 14 “away” populations ($PC1_{euc}$ = precipitation, $PC2_{euc}$ = heat, and $PC3_{euc}$ = variability), as well as the overall PC index, are presented in Supplementary Table S7. Smaller values indicate home-site climate that is similar to STL, while larger values indicate climatic dissimilarity.

Geographic distance and climate PC index were roughly equivalent predictors of fitness variation across populations for relative growth (Figure 3a, c). In contrast, climate PC index was a better predictor of variation in reproductive output than geographic distance; the R^2 value increased from 0.24 in the geographic distance model to

0.48 in the PC index model ($P = 0.003$; Figure 3b, d). Additionally, Climate PC index was highly correlated with geographic distance ($R^2 = 0.66$, $P = 0.0001$; Supplementary Figure S10).

Linear Mixed Models

The highest ranking models for the 2 fitness measures contained different parameters. Single parameter models best explained the relative growth in vegetative tissue, with home-site temperature variability (“variability”) containing the most predictive value, followed by maximum summer temperature (“heat”) (Table 3, Supplementary Table S8). “Precipitation” was the least important climatic parameter for relative growth, and adding “cyanotype” as a parameter did not improve relative growth models. Parameter weights across models

paralleled model rankings: variability (0.52), heat (0.42), precipitation (0.19), and cyanotype (0.00).

Inflorescence count was best explained by the model including precipitation + heat + cyanotype, and the addition of cyanotype improved models in all cases (Table 3, Supplementary Table S8). Parameter weights for heat and precipitation were 0.74 and 0.62 across inflorescence count models. In contrast to relative growth models, variability had the lowest parameter weight (0.45) for predicting inflorescence count, and cyanotype the highest (1.00), where Acli (the locally favored cyanotype) and AcLi cyanotypes were associated with increased fitness (Supplementary Table S8). This suggests a reproductive fitness advantage in the St. Louis climate for plants that produce cyanogenic glucosides. For the most important climatic parameters in all models, slopes were negative, indicating that home-site climate dissimilarity along those axes has negative effects on vegetative survival in St. Louis (Supplementary Table S8). Additional details for the top ranking models are presented in Supplementary Table S8.

Table 2. Results of the PCA for home-site climate variables during the growing season

Bioclim variable	PC1 (44%)	PC2 (24%)	PC3 (16%)
AnnMeanTemp	-0.318	-0.209	0.086
AnnPrecip	-0.313	0.217	-0.136
AnnTempRange	0.285	-0.198	-0.258
Apet	-0.222	-0.375	0.103
Isothermality	-0.212	-0.146	0.401
MaxT_warmM	-0.107	-0.456	0.035
MeanT_dryQ	-0.230	0.011	0.345
MeanT_warmQ	-0.221	-0.349	-0.113
MeanT_wetQ	-0.135	-0.194	-0.405
MeanTempRange	0.097	-0.411	0.196
Precip_dryM	-0.303	0.025	-0.217
Precip_dryQ	-0.305	0.048	-0.223
Precip_warmQ	-0.238	-0.031	-0.301
Precip_wetM	-0.272	0.254	0.025
Precip_wetQ	-0.264	0.284	0.005
PrecipSeasonality	0.190	0.103	0.261
TempSeasonality	0.253	-0.106	-0.380

Bold font indicates the 4 most highly correlated variables for each of the top 3 PCs. Italicized values correspond to maps in Supplementary Figure S9.

Discussion

Local adaptation in white clover has long been apparent from observations of repeatedly evolved clines in cyanogenesis. Less understood is the importance of this particular phenotype for overall plant fitness across varied climates. In this study, we evaluated the extent to which North American white clover populations exhibit local adaptation with respect to geographic or climatic distance from a central US common garden site, and we assessed the importance of cyanogenesis for predicting vegetative growth and reproductive output. We detect clear correlations between source population location and both of these fitness measures, with climatic distance the better predictor of reproductive output (Figure 3b, d). While cyanogenic plants showed marginally lower herbivore leaf damage (Figure 4), this effect did not translate into a fitness advantage at the common garden site (Supplementary Figure S8c,d). However, linear mixed modeling suggests that the cyanogenesis polymorphism may play some role in local adaptation for reproductive output (Table 3). Below, we discuss the implications of these findings for white clover

Table 3. Linear mixed model comparisons for 2 fitness measures

Model	Parameters included	K	Relative growth models		Inflorescence count models	
			Δ AIC	Akaike weight	Δ AIC	Akaike weight
1	Precipitation	1	2.929	0.107	316.729	0.000
2	Heat	1	0.817	0.306	316.059	0.000
3	Variability	1	0.000	0.461	315.929	0.000
4	Cyanotype	1	17.309	0.000	3.677	0.052
5	Precipitation + heat	2	3.837	0.068	311.959	0.000
6	Heat + variability	2	4.753	0.043	315.069	0.000
7	Precipitation + variability	2	7.120	0.013	316.139	0.000
8	Precipitation + cyanotype	2	22.808	0.000	3.484	0.061
9	Heat + cyanotype	2	20.742	0.000	2.131	0.120
10	Variability + cyanotype	2	19.967	0.000	2.576	0.096
11	Precipitation + heat + variability	3	9.961	0.003	312.839	0.000
12	Precipitation + heat + cyanotype	3	23.811	0.000	0.000	0.348
13	Precipitation + variability + cyanotype	3	27.060	0.000	3.490	0.061
14	Heat + variability + cyanotype	3	24.761	0.000	1.981	0.129
15	Precipitation + heat + variability + cyanotype	4	29.983	0.000	1.261	0.185

Akaike information criterion (AIC)-related metrics are given for models with all combinations of 4 parameters. Bold values indicate the Δ AIC and Akaike weights for the top 3 models in each set, with the top models italicized. Additional information for the top models is provided in Supplementary Table S8.

local adaptation and more broadly in the context of local adaptation, climate change, and restoration ecology research.

Rapid Local Climatic Adaptation in White Clover

Our data provide strong evidence that North American white clover has adapted to local climate on a continental scale, with this evolution having occurred in the 500 years since its introduction from Europe. Similar rates of evolved climatic adaptation have been noted in other systems, including annual plant species (Franks et al. 2007), invasive plants experiencing range expansion (Colautti and Barrett 2013), and salmonid fishes (Fraser et al. 2011). In white clover, rapid evolution is likely facilitated by its very large population sizes, with the species showing a near-continuous distribution in lawns, roadsides, and pastures across much of mesic North America, as well as an abundance of standing genetic variation that reflects intentional, repeated introductions of this agriculturally important plant (Kjærsgaard 2003). Such rapid evolution is promising in the face of climate change; however, the rapidity by which clover is able to evolve may be less generalizable to rare or range-restricted species with smaller population sizes and less genetic variation for selection to act upon (Franks et al. 2013).

While we find correlations between average population fitness and both geographic and climatic distance in the St. Louis common garden (Figure 3), climatic distance is a better predictor of fitness variation, particularly for reproductive output (Figure 3b, d). The strong correlation that we observe between geographic and climatic distance (Supplementary Figure S10) is likely largely a reflection of the central location of the common garden site in relation to population samples and the way Climate PC index was calculated (Figure 1, Supplementary Table S7). Across this continental scale, the key climatic variables show relatively smooth gradations (Supplementary Figure S9) and summing the *PC₁₋₃* distances to calculate climate PC index thus generated similar values for populations from similar geographic distances (Supplementary Table S7). For example, Duluth, MN (DMN) and Gainesville, FL (GFL) had similar climate PC indexes (8.039 and 8.437, respectively), but they differ climatically from STL in different ways. Duluth is wetter (*PC₁₋₂*) and colder (*PC₂₋₃*) than STL, while Gainesville is wetter (*PC₁₋₂*) and shows less variability in temperature (*PC₃*). Thus, the relationship between geographic and climatic distance is context-dependent, and geographic distance from the source population may not be the best predictor of fitness in general. These findings corroborate previous studies suggesting that restoration efforts are best advised to focus on environmental similarity when selecting individuals to transfer between habitats (Raabová et al. 2007; Lawrence and Kaye 2009; Noël et al. 2011; Forrester et al. 2013).

A key limitation of this study is the lack of reciprocal common garden sites for fitness comparisons. Identification of “reciprocal home-site advantage” in 2 or more locations is often considered the definitive test for demonstrating local adaptation (Kawecki and Ebert 2004). On the other hand, previous single-site studies have provided compelling evidence for local climatic adaptation in plants (e.g., Rutter and Fenster 2007; Preite et al. 2015; Peterson et al. 2016). The relatively large number of populations sampled in the present study and the clear evidence of climate-associated fitness variation that we detect across populations (Figures 2 and 3; Supplementary Figures S6 and S7; Table 3) lend further support to our conclusion that local climatic adaptation is pervasive in white clover. The results of this study are also entirely consistent with inferences from cyanogenesis cline studies indicating that the species

repeatedly locally adapts across climatic gradients. Nonetheless, future multi-site common garden experiments in white clover would undoubtedly be valuable and could be especially useful for examining fitness-related traits not considered here—for example, flowering phenology, a critical trait for local climatic adaptation in many plant species (e.g., Weinig et al. 2002; Verhoeven et al. 2008; Buckler et al. 2009; Anderson et al. 2011, 2013; Friedman and Willis 2013).

Another limitation of the study is that we did not consider the potential impacts of competition on fitness variation. By trimming and weeding, we eliminated both conspecific and heterospecific plant competition. It is thus possible that regional variation in competitive ability exists that we did not capture in this study. Additionally, we did not consider the impact of soil nutrients or microbes on fitness, which may be particularly important for legumes such as white clover that interact with local soil *Rhizobia* (Macel et al. 2007). Follow-up studies would be valuable for examining both of these factors.

The Effects of Cyanogenesis on Fitness

While our data suggest that cyanogenic (AcLi) plants experience marginally less herbivore leaf damage than acyanogenic plants in St. Louis (Figure 4, Supplementary Figure S8b), this advantage did not result in higher fitness for either fitness measure examined here (Supplementary Figure S8c,d). We detected very low herbivory overall in the common garden (10–12% of leaves on average showed some amount of discernible herbivore damage) (Supplementary Figure S8a). By comparison, Kooyers et al. (2014) found that on average, 25–30% of individual leaflets displayed some amount of herbivore leaf damage across 4 natural populations located south of St. Louis in Tennessee, Arkansas, and Oklahoma. Thus, our results are potentially consistent with lower herbivore damage in the central United States than in southern US populations, as would be expected if variation in herbivore abundance drives the evolution of cyanogenesis clines. However, the 2 studies may not be directly comparable given that the present study examined herbivory in non-local genotypes and in a different year. In contrast to our findings, Thompson and Johnson (2016) quantified mean herbivore leaf damage by estimating overall percent damage per leaf on plants in a more northern common garden (Toronto, Canada) and found leaves experienced 35.7% and 23.8% herbivore damage on average during early and late season surveys, respectively. That high level of herbivory is somewhat unexpected given low frequencies of cyanogenic plants in most northern populations (but see Thompson et al. 2016).

Interestingly, rather than detecting a fitness advantage for cyanogenic plants in the common garden location, we instead found that the cyanotype that is most common in local wild populations (AcLi) showed the highest mean fitness for both relative growth and reproductive output, although this trend was not statistically significant (Supplementary Figure S8c,d). To our knowledge, these are the first data to establish a relationship between high cyanotype frequency in local wild populations and high fitness of nonlocal plants of the same cyanotype in that region. The AcLi cyanotype produces cyanogenic glucosides but lacks the enzyme required for HCN release. Growth chamber experiments suggest that this cyanotype shows differentially high reproductive fitness under simulated drought conditions when nitrogen is limited (Kooyers et al. 2014). Consistent with that finding, studies in sorghum and other species indicate that cyanogenic glucosides can be metabolized through noncyanogenic pathways and are likely beneficial as a nitrogen reserve under drought stress conditions (Møller 2010; Kooyers 2015). Thus, the slightly

elevated fitness of Acli that we detect in the common garden might be a reflection of differential success during the dry, hot days of the peak growing season in the central United States.

Predictive Abilities of Cyanogenesis Versus Climatic Parameters for Fitness

Model averaging indicated that alternative climatic parameters are the best predictors of different aspects of fitness in white clover. Our findings agree with previous studies showing that survival and growth-related traits respond more strongly to temperature than precipitation (Moles et al. 2014; Preite et al. 2015), whereas water availability and precipitation are particularly important for flowering, especially during the driest portions of the growing season (Prieto et al. 2008; Samis et al. 2012). Additionally, we documented striking variation across populations in their rate and magnitude of flowering in response to bouts of precipitation during the reproductive season. This result suggests that it is important to consider not only the total reproductive output but also the tempo of output relative to periodic environmental cues when assessing local adaptation. Furthermore, the predictive nature of chemical defense (in this case, cyanotype) for floral production suggests that knowledge of locally favored adaptive traits, in addition to climate similarity, can help to inform restoration ecologists in selecting the most appropriate individuals for transplantation efforts.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

We have deposited the primary data underlying these analyses as follows:

- Sample coordinates, geographic distance to the common garden site and cyanotype information for all genotypes used in this study: Supplementary Table S1
- Block and position assignments, source population climate information, cyanotype, and fitness data for the 483 plants included in the common garden experiment: Supplementary Table S3
- VCF files for GBS data: Dryad doi: 10.5061/dryad.j3ck6
- Cyanotype data for plants included in the germination experiment: Supplementary Table S6
- Raw digital photos used in quantifications of vegetative tissue area: Dryad doi: 10.5061/dryad.j3ck6

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