

Geographic variation in flowering phenology in *Boechera stricta*:
testing for adaptive response to climate across the species range

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Abstract

As climate continues to shift, species existing over broad and variable ranges must exhibit the ability to adapt functional traits to novel environmental conditions. In this study, I used a large-scale common garden experiment to examine the variation in flowering time of 137 *Boechnera stricta* (Brassicaceae) genotypes, representing populations from across the species range. The experiment found variance in date of first open flower among genotypes, with those originating at northern and high-elevation (i.e. shorter growing season) sites flowering sooner than those from southern and low-elevation sites. These results suggested that adaptation of flowering phenology has occurred on a fine scale and is driven by local environmental conditions historically experienced at each genotype's site of origin. Broader-scale phenological adaptation was investigated by partitioning the genotypes into six population groups based on genetic divergence over approximately 2,000 generations; however, evidence for adaptation on this scale was not found. The finding that *B. stricta* has evolved on a fine scale in accordance with the local environment of origin may demonstrate the species' ability to continue adapting to changing climatic conditions.

Introduction

The timing of plant life cycle events (phenology), such as the date of first open flower production, is considered especially responsive to the environmental shifts associated with anthropogenic climate change (Prendeville *et al.* 2013). Local adaptation should cause each population to evolve advantageous phenological traits based on the environmental conditions in the location in which it has evolved. If a population cannot adaptively keep pace with changing climate, traits that previously provided an advantage may become unfavorable under new climate scenarios, therefore causing a decline in reproductive success and fitness (Kawecki & Ebert, 2004). In addition, on a systems scale, species across trophic levels may differ in their physiological or life-history responses to novel environmental conditions, disrupting previously synchronous relationships, such as those involving pollination, seed dispersal, food acquisition, or habitat structuring (Parmesan, 2006).

Historical phenology data for many northern plant taxa show accelerated flowering time when grown under controlled conditions, while plants from lower latitudes show slower reproductive development (Prendeville *et al.* 2013; Paccard *et al.* 2013). A recent study Anderson and Gezon (2014) conducted at the Rocky Mountain Biological Laboratory (RMBL; Gothic, Gunnison County, Colorado) showed both plasticity and heritable genetic variation in flowering time among 24 discrete populations of *Boechnera stricta* (Brassicaceae) from sites between 2869 and 3682 m, but comparative studies quantifying differences in phenology between geographically distinct populations on a range-wide scale have yet to be conducted. I utilized a large-scale common-garden approach to test for evidence of contemporary adaptation to climate, which can help to elucidate a species' ability to adapt under projected novel climate conditions (Pratt & Mooney 2013). I tested the hypothesis that northern and high-elevation genotypes of *B. stricta* would exhibit more rapid reproductive phenology than southern and low-elevation genotypes when grown in a common garden, indicating possible adaptive evolution of these traits to the local environmental conditions of their location of origin. Comparing phenological trait variation among genotypes originating from across a range-wide latitudinal gradient, when grown in a common garden, can provide insight into future population performance (Paccard *et al.* 2013). Specifically, I investigated phenological trait variation in *Boechnera stricta* to address the following questions:

1. How does flowering phenology vary among populations across the species range?
2. What is the relationship between evolutionary origin and intraspecific phenological heterogeneity?
3. If evidence for phenological adaptation exists, on what scale is it occurring?

Methods

Study System and Experimental Design

Study Organism

Boechera stricta (Brassicaceae) is a short-lived perennial forb, native to western North America, and found over a large geographic and climatic range. *B. stricta* is primarily diploid, 95% self-pollinating, and is a close relative of *Arabidopsis thaliana* (Song *et al.* 2009). Intraspecific populations existing over an elevation gradient often adapt functional traits in accordance with variation in environmental conditions (Anderson *et al.* 2012). Some *Boechera* populations occur in environments known to have been undisturbed by agriculture or forestry for ~3,000 years (Brunelle *et al.* 2005). While global climate change has influenced most terrestrial ecosystems, the lack of direct habitat alteration by humans makes *Boechera* a prime candidate for investigating evolutionary questions in historically-relevant habitats (Rushworth *et al.* 2011).

Common garden experiment

In this study I used 277 fully sequenced accessions of *B. stricta* genotypes originating from seeds collected from populations across the species range (Fig. 1). Each genotype was inbred under controlled conditions at the Duke University greenhouse for at least one generation to eliminate maternal effects. Five individuals of each genotype (N=1385) were germinated at Duke, grown under uniform greenhouse conditions for 10 weeks, and vernalized in a cold room at 4°C for 8 weeks. Rosettes were shipped to Western State Colorado University (WSCU; Gunnison, Gunnison County, Colorado) in April 2015, where they continued vernalization under field conditions at WSCU for an additional 4 weeks. In late May, the plants were transplanted into 4-inch pots in a standard potting soil (Scotts Miracle-Gro) and arranged in a randomized complete block design in Maxfield Meadow, 0.5 miles south of Rocky Mountain Biological Laboratory (Fig. 2). The garden layout consisted of 5 blocks, each containing 3 rows with approximately 115 pots per row (5×23). Wire cages were constructed around each row of plants to discourage damage from macroherbivores. All plants received equal amounts of water as necessary to prevent soil drying, and were censused every other day throughout the reproductive season to record the date on which each plant produced its first open flower.

Climate data from between the years 1950 to 2000, including mean annual temperature and annual precipitation, were compiled for each genotype source site using the WorldClim database. These data were used to correlate environmental variables with latitude and elevation to discern the influence of climate and geography on flowering date across the species range (Paccard *et al.* 2013).

Statistical Analysis

All analyses were carried out using JMP 12 statistical software (SAS Institute, Inc.).

How does flowering phenology vary among populations across the species range?

To analyze the how flowering phenology differed across the species range, the genotypes were partitioned into six population groups based on a principal component analysis (PCA) performed by the Mitchell-Olds lab at Duke University. The PCA used ~100,000 single nucleotide polymorphisms to separate genotypes into groups based on historical genetic divergence and structuring. The six population groups were assigned names based on where the majority of the member genotypes clustered geographically (Western [WES], Northern [NOR], Central [CEN], Colorado [COL], Utah [UTA], and Admixed [ADM]; Fig. 3). The mean flowering date was calculated for each genotype by averaging the dates on which all individuals of that genotype produced their first flower. A one-way ANOVA was used to compare mean flowering dates among the six population groups based on the average flowering date of each genotype within that group. Finally, a Tukey HSD post-hoc test was used to determine which of the six population groups differed significantly from one another in pairwise comparisons.

What is the relationship between geographic origin and intraspecific phenological heterogeneity?

To test the relationships between environmental and geographic variables, simple regression analyses were performed to examine how mean annual temperature, annual precipitation, latitude, longitude, and elevation were related to each other individually. A scatterplot matrix was used to identify the strongest relationships based on each Pearson's correlation coefficient.

A standard least squares model was used to construct a multiple regression to test how average flowering date varied in response to latitude, longitude, elevation, annual mean temperature, and annual precipitation. The model's significant predictor variables ($P < 0.05$) were then used to create the most parsimonious restricted maximum likelihood (REML) model to determine the statistical significance of each effect. From this model, a multidimensional surface plot was built to illustrate the relationship between the predictor variables, their interactions with one another, and their influence on average flowering date.

If evidence for phenological adaptation exists, on what scale is it occurring?

A comparison of divergence at neutral molecular markers (F_{ST}) to divergence at quantitative functional traits (Q_{ST}) was used to determine whether natural selection or neutral genetic drift was driving variation in flowering time among the PCA-determined population groups (Leinonen *et al.* 2013). The neutral genetic divergence at all ~100,000 loci was calculated as a distribution of F_{ST} values across the genome for all pairwise comparisons of the six population groups. The means and standard errors of all pairwise F_{ST} distributions were averaged to determine the level of overall neutral genetic differentiation across the species range. Q_{ST} was determined by calculating variance components from a linear mixed effects REML model with flowering date as the response variable. Block, row nested within block, flowering time observer, population group, and genotype ID nested within population group were included as random effects to calculate the percent of variance in flowering date accounted for among population

groups vs. individuals within population groups. The percent of variance in flowering date accounted for by population group and individual within population group were summed to calculate Q_{ST} for that trait. The Q_{ST} value was then compared to the F_{ST} value to see if it was larger by at least two standard errors of mean F_{ST} , thereby implying that the phenological differentiation observed among population groups was the result of adaptive processes rather than neutral processes (Merilä & Crnokrak 2001).

Results

Common garden experiment

Deer browsing during the first week of June, prior to the construction of protective cages, reduced the initial sample size of 1303 plants to 1134 surviving plants, with 882 of those receiving no damage. Between the months of June and July, 804 individual plants had flowered, representing 137 genotypes with 3 or more replicates flowering. Only plants that had flowered prior to deer browsing, or had not been damaged by the browsing event, were included in the flowering date data set.

How does flowering phenology vary among populations across the species range?

Mean flowering date (Julian day) varied significantly among population groups ($N=137$, $F\text{-ratio}= 5.5891$, $P= <0.0001$). Post-hoc testing indicated that three populations groups were driving overall significant differentiation (Admixed-North, difference of 5.17 days, $P= 0.0003$; Admixed-Utah, difference of 5.11 days, $P= 0.0031$, North-West, difference of 4.33 days, $P= 0.0302$; Fig. 4).

What is the relationship between geographic origin and intraspecific phenological heterogeneity?

Simple regression analysis indicated statistically significant ($P<0.01$) Pearson correlation coefficients of varying strengths for all but four of the possible pairwise relationships between the each geographic, environmental, and phenological variables. The strongest of the relationships ($r > 0.35$ or $- 0.35$) were as follows: elevation-latitude, elevation-longitude, annual mean temperature-elevation, annual precipitation-elevation, mean flowering date-elevation, and mean flowering date-annual mean temperature (Table 1).

Table 1. Pearson correlations coefficients for each pair of geographic, environmental, and phenological variables. The strongest of the relationships are indicated by the asterisk (*): elevation-latitude ($r = -0.6808$), elevation-longitude ($r = -0.4209$), annual mean temperature-elevation ($r = -0.4871$), annual precipitation-elevation ($r = 0.4523$), mean flowering date-elevation ($r = -0.4003$) and mean flowering date-annual mean temperature ($r = 0.3851$). Correlations were calculated using simple regression analyses.

	Latitude	Longitude	Elevation	Annual Precipitation	Annual Mean Temp.	Mean Flowering Date
Latitude	1.0000	-0.5674	-0.6808*	-0.3204	-0.2222	0.0704
Longitude	-0.5674	1.0000	0.4209*	0.1245	0.1463	-0.1221
Elevation	-0.6808*	0.4209*	1.0000	0.4523	-0.4871*	-0.4003
Annual Precipitation	-0.3204	0.1245	0.4523	1.0000	-0.2566	-0.1395
Annual Mean Temp.	-0.2222	0.1463	-0.4871*	-0.2566	1.0000	0.3851*
Mean Flowering Date	0.0704	-0.1221	-0.4003*	-0.1395	0.3851*	1.0000

The model testing the simultaneous influence of geographic and environmental variables on flowering date indicated that only elevation and latitude were significant predictor effects (Table 2). Non-significant effects were removed and the model was run testing the response of mean flowering date to elevation, latitude, and the interaction of elevation $\times$ latitude, resulting in the most parsimonious model (Table 3).

Table 2. The results of the initial multiple regression (standard least squares model) testing the simultaneous influence of all geographic and environmental variables indicated that only elevation and latitude were significant predictors of mean flowering date.

Effect	DF	Sum of Squares	F Ratio	P-Value
Elevation	1	205.86490	11.5209	0.0009*
Latitude	1	109.58757	6.1329	0.0146*
Longitude	1	8.14125	0.4556	0.5009
Annual mean temperature	1	6.73922	0.3772	0.5402
Annual precipitation	1	32.67433	1.8286	0.1786
$R^2 = 0.246$				

* $P < 0.05$

Table 3. The results of the most parsimonious multiple regression analysis (standard least squares model) indicated that elevation, latitude, and elevation $\times$ latitude were significant predictors of mean flowering date.

Effect	DF	Sum of Squares	F Ratio	P-Value
Elevation	1	994.638	51.516	< 0.0001*
Latitude	1	160.904	8.3357	0.0044*
Elevation $\times$ Latitude	1	145.171	7.5190	0.0068*
$R^2 = 0.271$				

* $P < 0.05$

The three-dimensional surface plot (Fig. 5) illustrates the negative correlation between flowering date with both elevation and latitude. The elevation $\times$ latitude interaction is evident in the plot's surface curvature, illustrating the strong effect of elevation on latitude's influences on flowering date, as well as the less substantial effect of latitude on elevation's influence on flowering date.

If evidence for phenological adaptation exists, on what scale is it occurring?

Overall neutral differentiation (F_{ST}) among population groups was determined by averaging each pairwise comparison of variance at neutral loci, resulting in a value of 0.122 ± 0.0062 . The REML model determined variance in flowering time due to population group to be 0.01408 and variance due to individual plant nested within group to be 0.0944. The two variance percentages were summed, resulting in an overall Q_{ST} value for flowering date of 0.108. F_{ST} and Q_{ST} values were determined to not be statistically different based on the calculated Q_{ST} value for flowering date falling within two standard errors of the average F_{ST} .

Discussion

Examining intraspecific trait variation among geographically discrete populations, and the possible drivers of that differentiation, can help elucidate the mechanisms and scale of past and future adaptation. The common garden experiment found that *Boechera stricta* populations originating from across the species range varied substantially in flowering phenology when grown together under uniform conditions, with a magnitude of variation between earliest and latest flower genotypes of 24 days. As hypothesized, the average date on which each genotype produced its first open flower was most significantly influenced by the elevation and latitude of the place from which it originated; with those from northern and/or high elevation sites flowering before those from southern and/or low elevation sites. The observed genotype-environment association suggests fine-scale phenological adaptation to the local environment of origin (Mitchell-Olds & Schmitt, 2006). However, the magnitude of variation in flowering date among genetically divergent groups of genotypes (population groups) was not significantly greater than neutral genetic divergence among populations, therefore adaptation on a broader scale was not inferred.

By partitioning the genotypes into genetically divergent population groups, I was able to test for broad-scale adaptation, thereby determining whether or not flowering time might be driven by historic neutral genetic structuring rather than adaptation to the local environment in which each genotype was found (Lee & Mitchell-Olds 2011). The analysis of variance among

population groups indicated that only 3 out of 15 pairwise comparisons of mean flowering date between groups were significantly different. This result suggested that variation in flowering phenology across the species range was likely occurring on a scale finer than the broadly dispersed population groups to which each genotype belonged. The further investigation of broad-scale adaptation by way of Q_{ST} - F_{ST} comparison indicated that variance in flowering date among population groups did not exceed neutral expectations (e.g. the extent of variation due to genetic drift) therefore did not imply directional selection (Leinonen *et al.* 2013). This result further strengthened the suggestion that adaptation for flowering time is not strongly driven by the historic genetic divergence that determined the population groups, and is likely occurring on a finer scale and by more local influences.

Genotype-level analysis provided better indications of the drivers of phenological adaptation and the scale at which it might be occurring. By examining the relationship between geographic and climate variables, it became apparent that environmental conditions across the species range were dynamic and most strongly correlated with source site elevation. As expected, sites at higher elevations historically received more precipitation and experienced lower mean temperatures. The negative correlation between latitude and elevation indicated that many of the lower elevation sites were clustered near the northern extent of the collection range, resulting in latitude being negatively correlated with both temperature *and* precipitation, contrary to trends observed in less topographically dynamic regions (Prendeville *et al.* 2013). This relationship emphasized the need to incorporate the interaction between elevation and latitude and their collective influence on flowering date into statistical models.

Mixed effects modeling indicated that elevation, latitude, and elevation $\times$ latitude had the only significant influence on flowering date when all geographic and environmental variables were included as effects. As previous studies by Anderson *et al.* (2012) and Prendeville *et al.* (2013) have shown, phenology is highly responsive to temperature and precipitation, and because these two variables are both captured by their correlations with geographic variables, elevation, latitude, and the interaction between the two became the strongest predictors of flowering time. The model confirmed that the genotypes from the most northern regions and highest elevations flowered sooner than those from southern regions and lower elevations. From this we can infer that northern and high-elevation genotypes have adapted to shorter reproductive windows due to lower temperatures and increased annual precipitation (i.e. longer winters) than southern and low-elevation genotypes. Therefore, when grown in a common garden, the northern and high-elevation genotypes flower sooner relative to genotypes that have historically experienced more protracted growing seasons—results that are congruent with studies employing early snow removal manipulations to simulate longer reproductive windows (Anderson & Gezon 2014). This genotype-environment association suggests that flowering phenology has adapted to the discrete local environments in which each population has evolved, selecting for genotypes that time flowering to occur under what is presumed to be the most optimal conditions for reproduction.

The results of this study indicate that adaptation of flowering phenology in *B. stricta* is occurring on the fine-scale genotype level rather than being driven by historical genetic divergence and population structuring at broad geographic scales. The date on which a genotype flowered was dependent on the length of the growing season that it had historically experienced, inferred by its geographic origin and the historical environmental conditions of that origin. Fine-scale adaptability not only suggests the species has substantial tolerance to a wide array of

habitats and environmental conditions, but also increases the genetic diversity from which to select as novel environmental conditions evolve across the species range.

While this study has shown the ability of *Boechnera stricta* to adapt to past environmental change, ongoing and future studies investigating the role of phenotypic plasticity in helping to keep pace with the unprecedented rate at which climate is projected to shift will further elucidate future population performance.

Figures

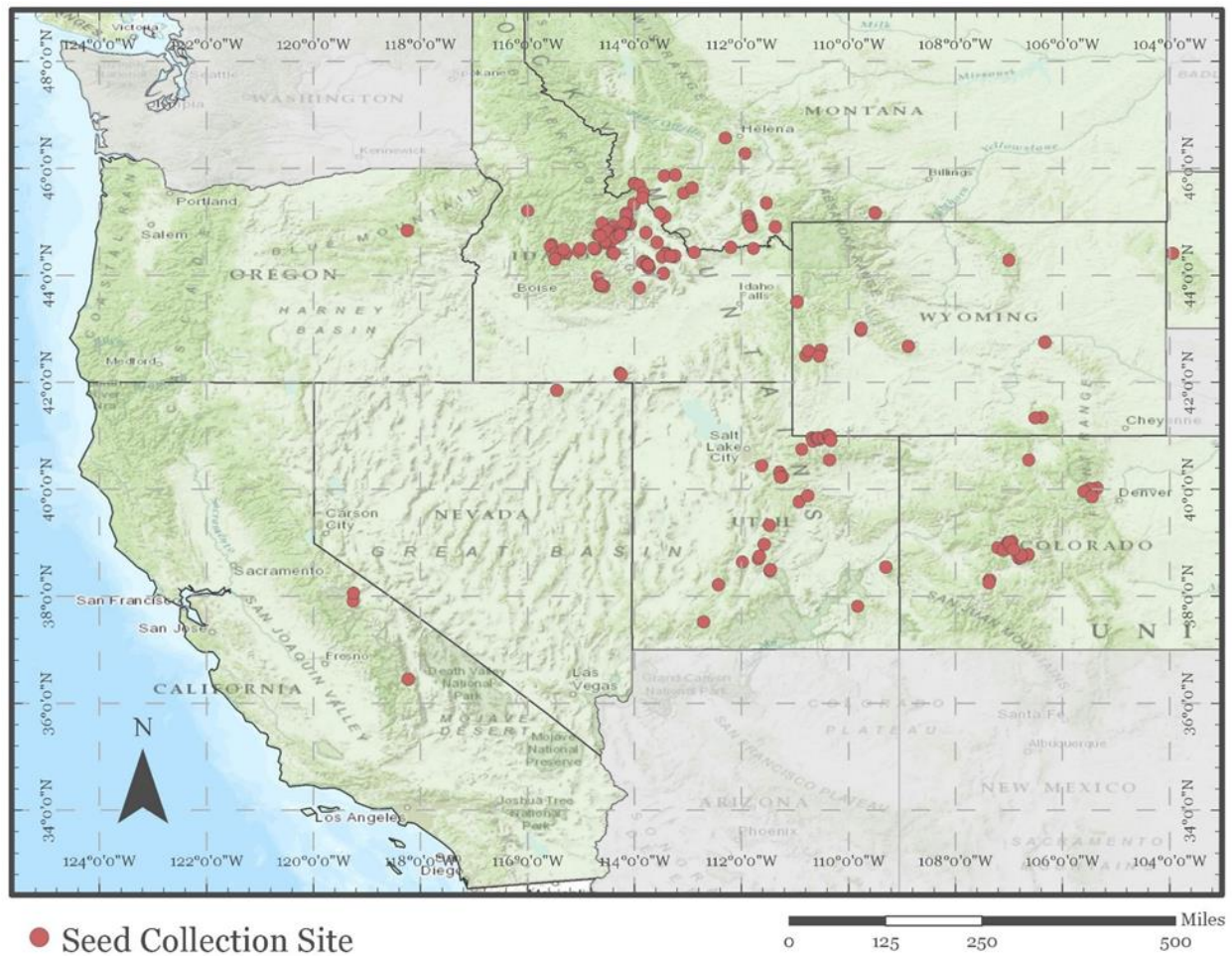


Figure 1. Map of the western United States showing the sites from which *Boechnera stricta* seeds were collected for use in the common garden experiment. Sites range in elevation from 1632 m to 3658 m, and in latitude from 36.449° (Sequoia National Park, CA) and 46.268° (Elkhorn, MT).



Figure 2. Common garden site, located approximately 0.5 miles south of Rocky Mountain Biological Laboratory in Gothic, Colorado. The garden was located 100 m west of County Road 317, at 2895 m, on a slope with easterly aspect and full sun.

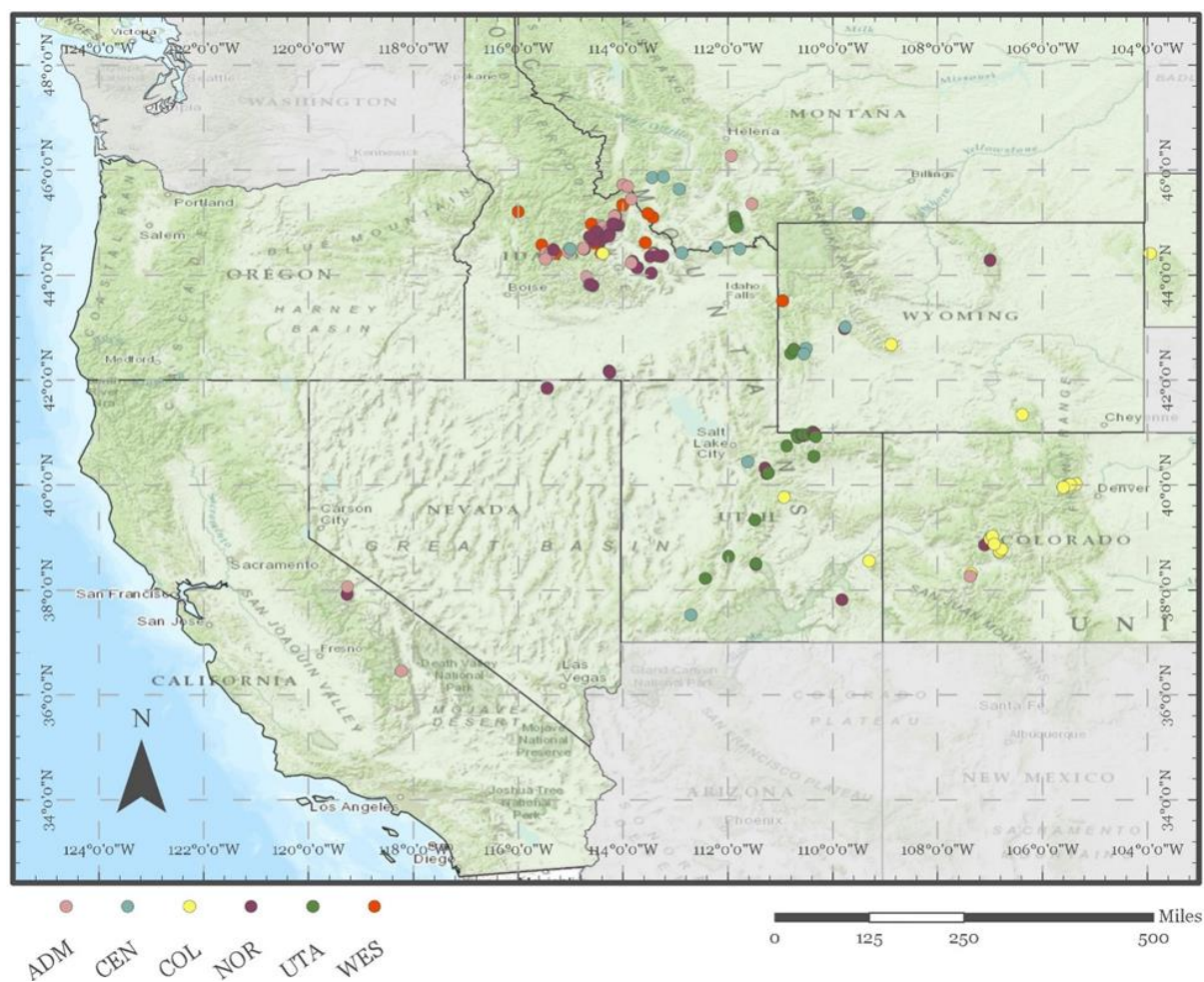


Figure 3. Map of the western United States indicating the PCA-determined population group to which each genotype belongs. The PCA used ~100,000 single nucleotide polymorphisms to partition genotypes into groups that have been genetically diverging for approximately 2,000 generations.

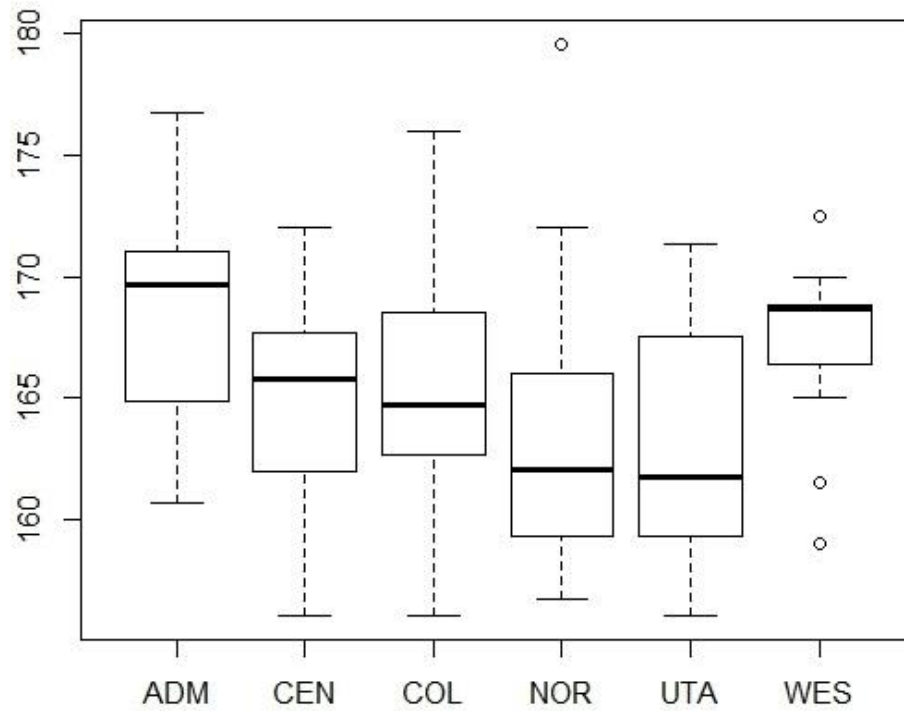


Figure 4. Analysis of variance among population groups indicated significant differences in average flowering date. Post-hoc test results revealed that pairwise comparisons between the Admixed [ADM]-North [NOR] ($P=0.0003$), Admixed-Utah [UTA] ($P=0.0031$), and North-West [WES] ($P=0.0302$) groups were responsible for the overall significant differentiation.

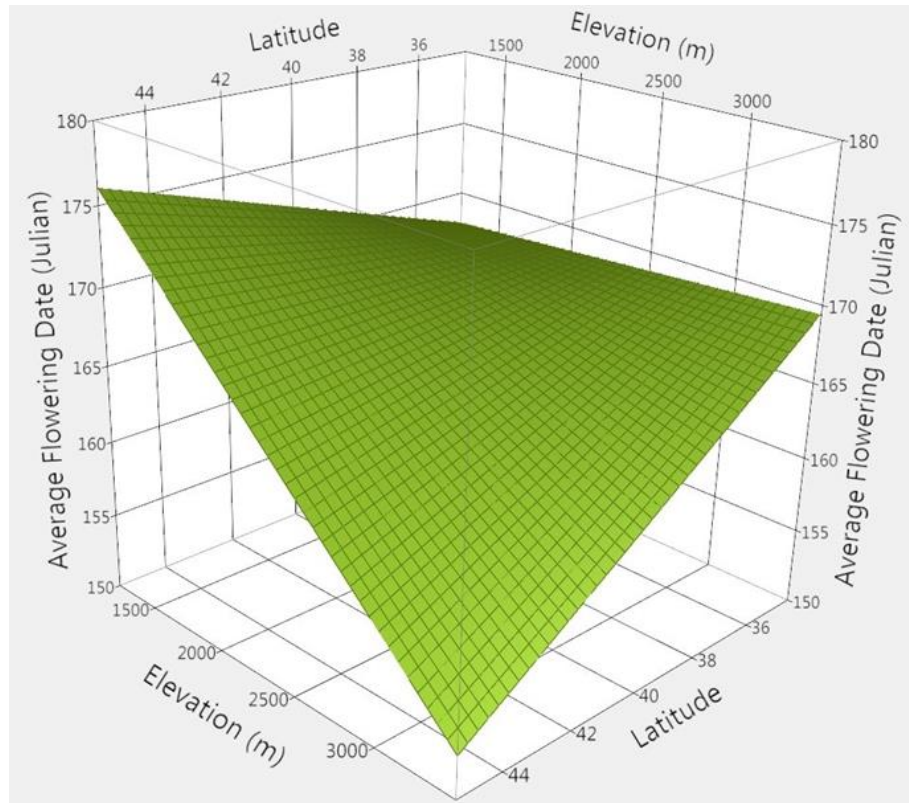


Figure 5. Three-dimensional surface plot depicting the effects of elevation of origin, latitude of origin, and the interaction of elevation $\times$ latitude on the average flowering date of a genotype when grown in a common garden. The elevation $\times$ latitude interaction is evident in the plot's surface curvature, illustrating that the effect that each has on the response variable is significantly influenced by the other.

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