**Methods**

***Plant Collection***

*Solanum carolinense* plants from three populations in Texas and two populations in Minnesota were collected between October 2019 and August 2020 (figure 1). The three southern populations were from Colin County, Texas near McKinney (Oil Patch: 33.173465 N, -96.615402 W; Reserve: 33.159962 N, -96.619011 W; and Cemetery: 33.173672, -96.615096 W). Each population consisted of between 10 and 50 plants in the fruiting stage of their life history. The Reserve population was located approximately 1.5 km from the Oil Patch and Cemetery populations which were adjacent to each other (figure 2). The two populations from the north were from Houston County, Minnesota and from here on will be referred to as plants from the northern region or Prairie Island (44.07959 N, -91.684545 W) and Frontenac (44.523056 N, -92.338611 W). These populations are separated by approximately 50 miles (figure 3). In Colin County TX, the average monthly low temperature is 18°C (65°F) and the average monthly high is 43°C (111°F). In Houston County, MN, the average, the average monthly low temperature is -14°C (7°F) and the average monthly high is 29°C (85°F).

*Solanum carolinense* is a rhizomatous, herbaceous perennial that disperses via sexual reproduction with a tomato-like fruit and by growing ramets from the rhizomes. Therefore, plants in close proximity may be genetically identical or ramets. To avoid sampling two plants of the same genotype, plants with a minimum inter-plant distance of 1 meter were collected. Collections involved digging up and cutting rhizome of at least 10 cm and placing them in ziplock bags. Rhizomes were stored in a cooler with blue ice and shipped to Fargo, where the collections were stored in a 4°C refrigerator. The rhizomes were potted in one-gallon containers and grown throughout the summer of 2020. In October, all above ground matter was cut and the rhizomes were again stored in a 4°C refrigerator to induce a period of dormancy.

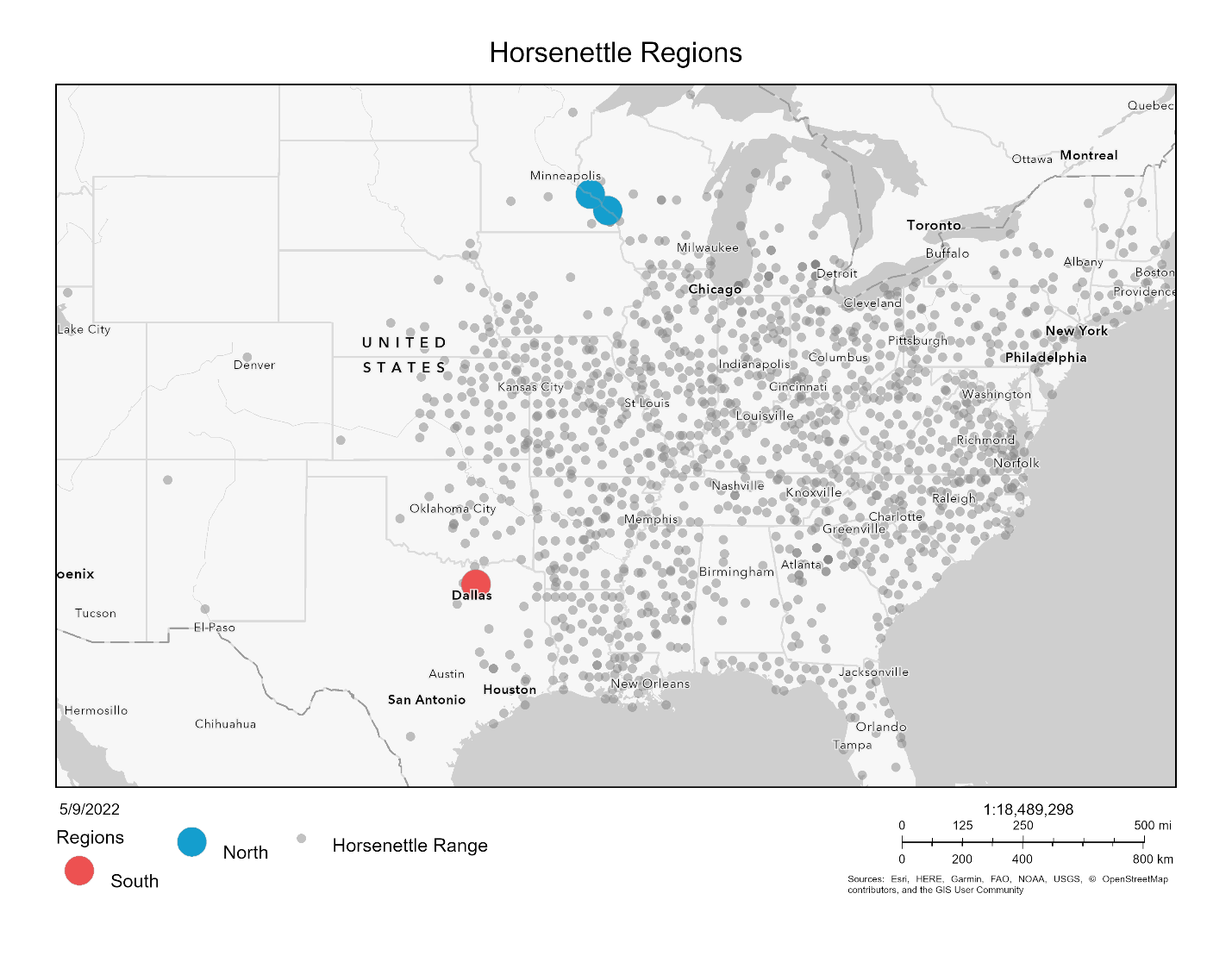
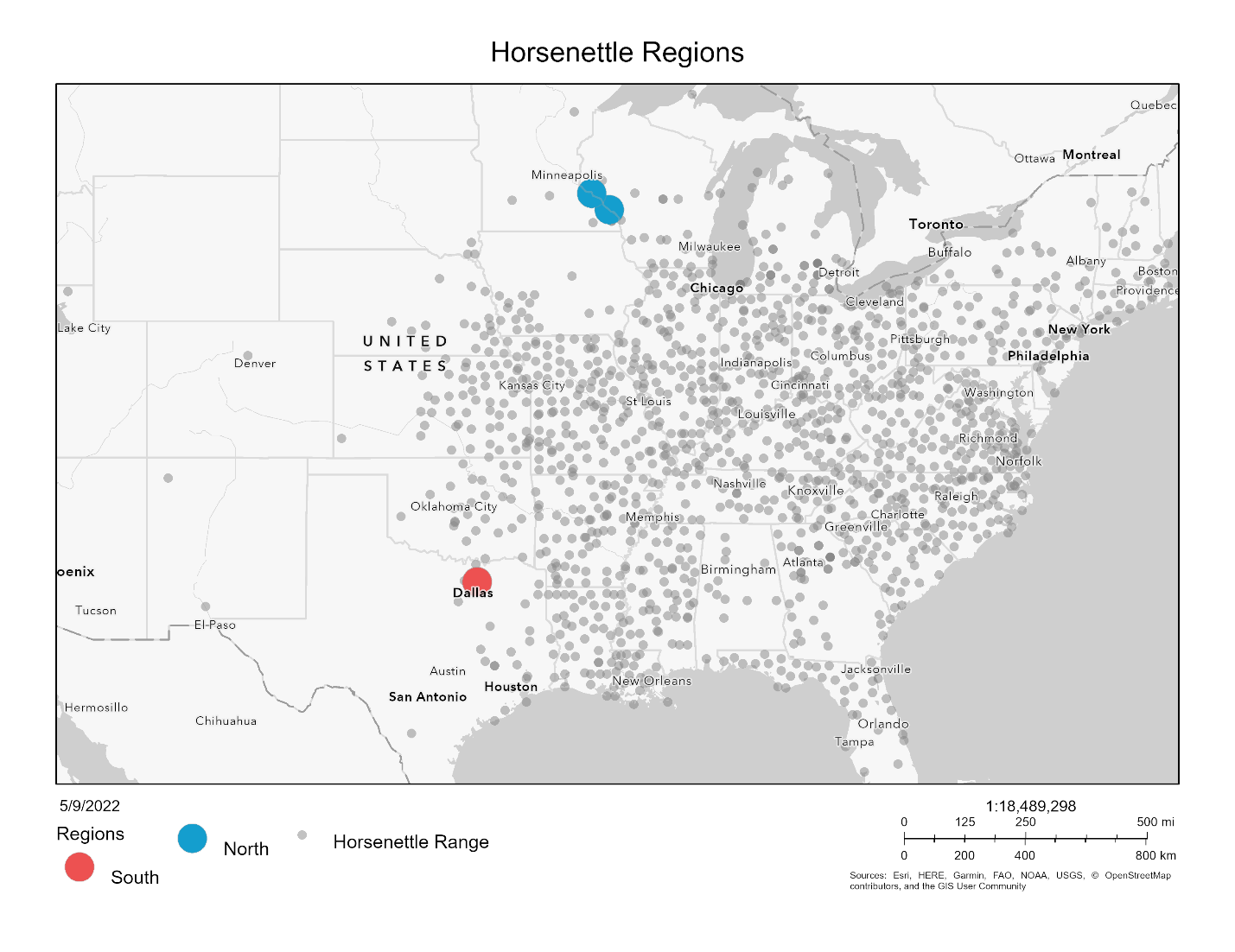
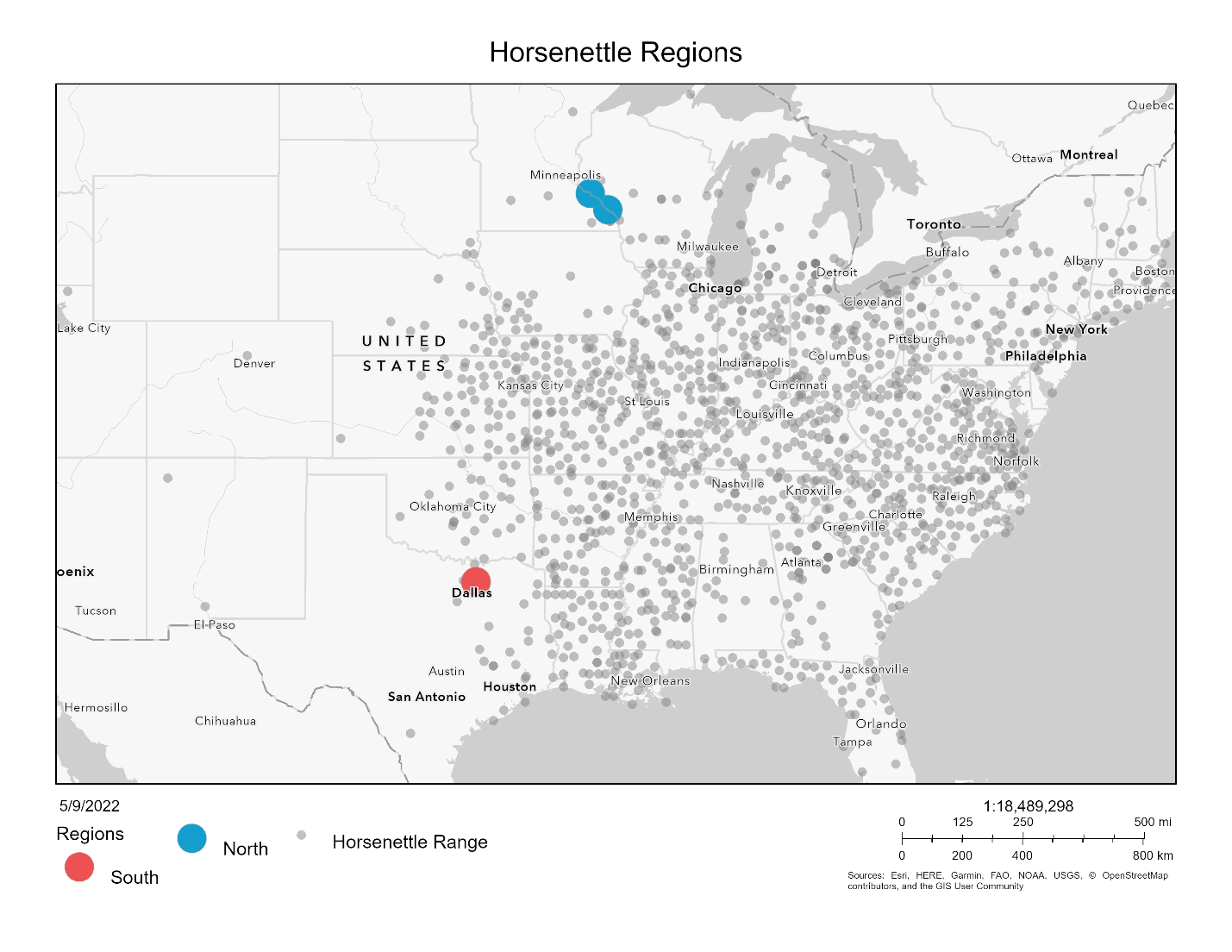


Figure 1. Map with collection site. Northern sites in blue and southern sites in red. Grey points indicate sites where *Solanum carolinense* was observed (data from EddsMaps).

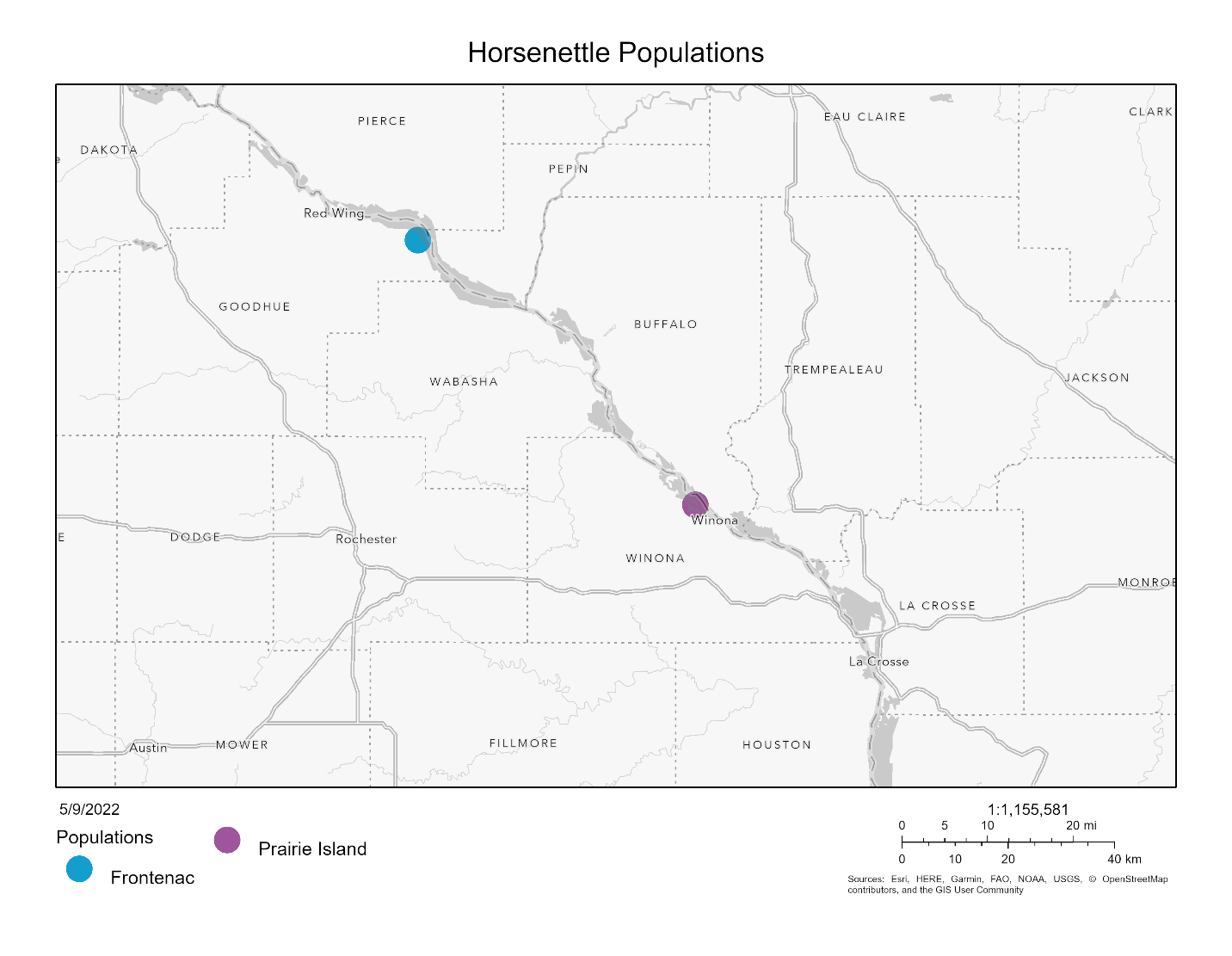
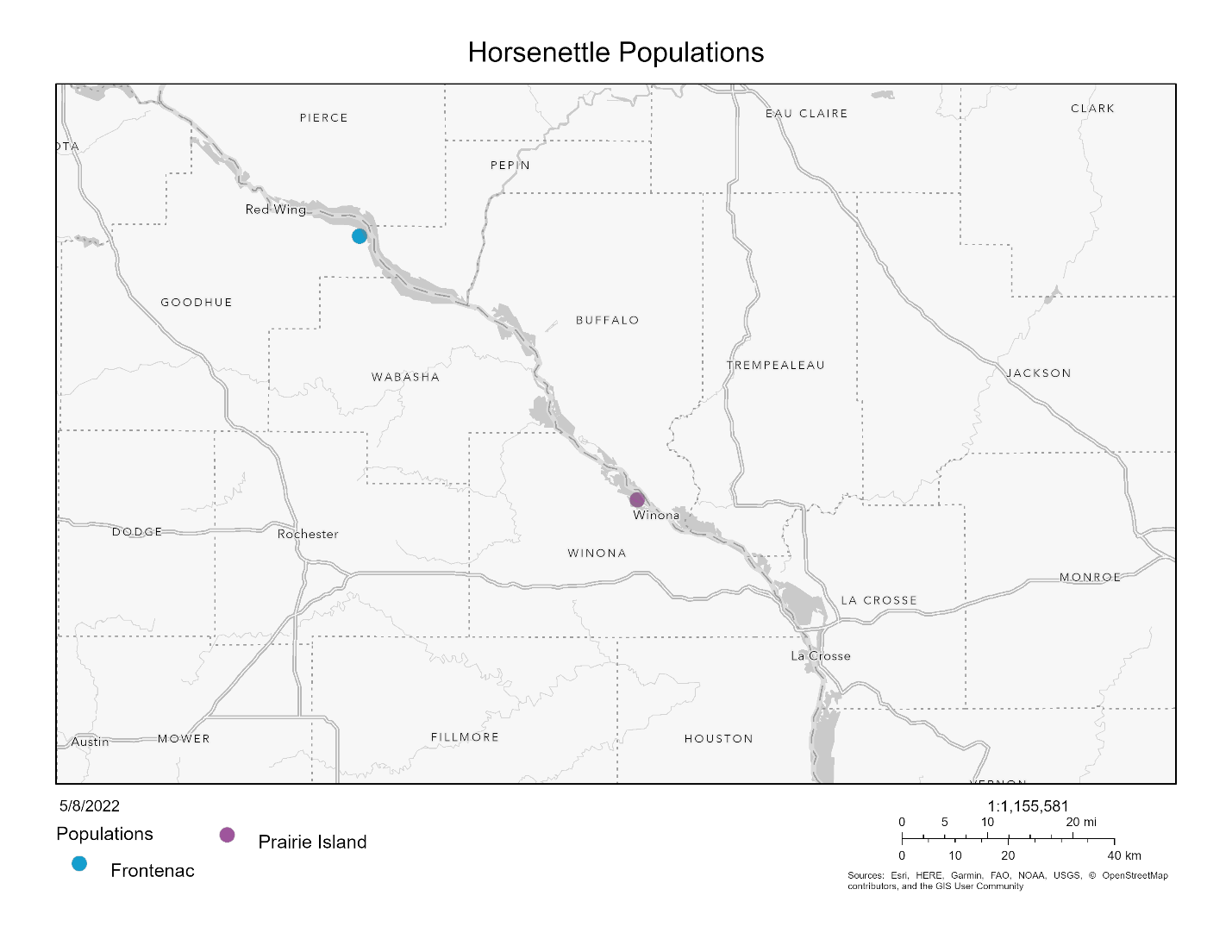


Figure 2. Populations in the northern region. Frontenac in blue and Prairie Island in purple.

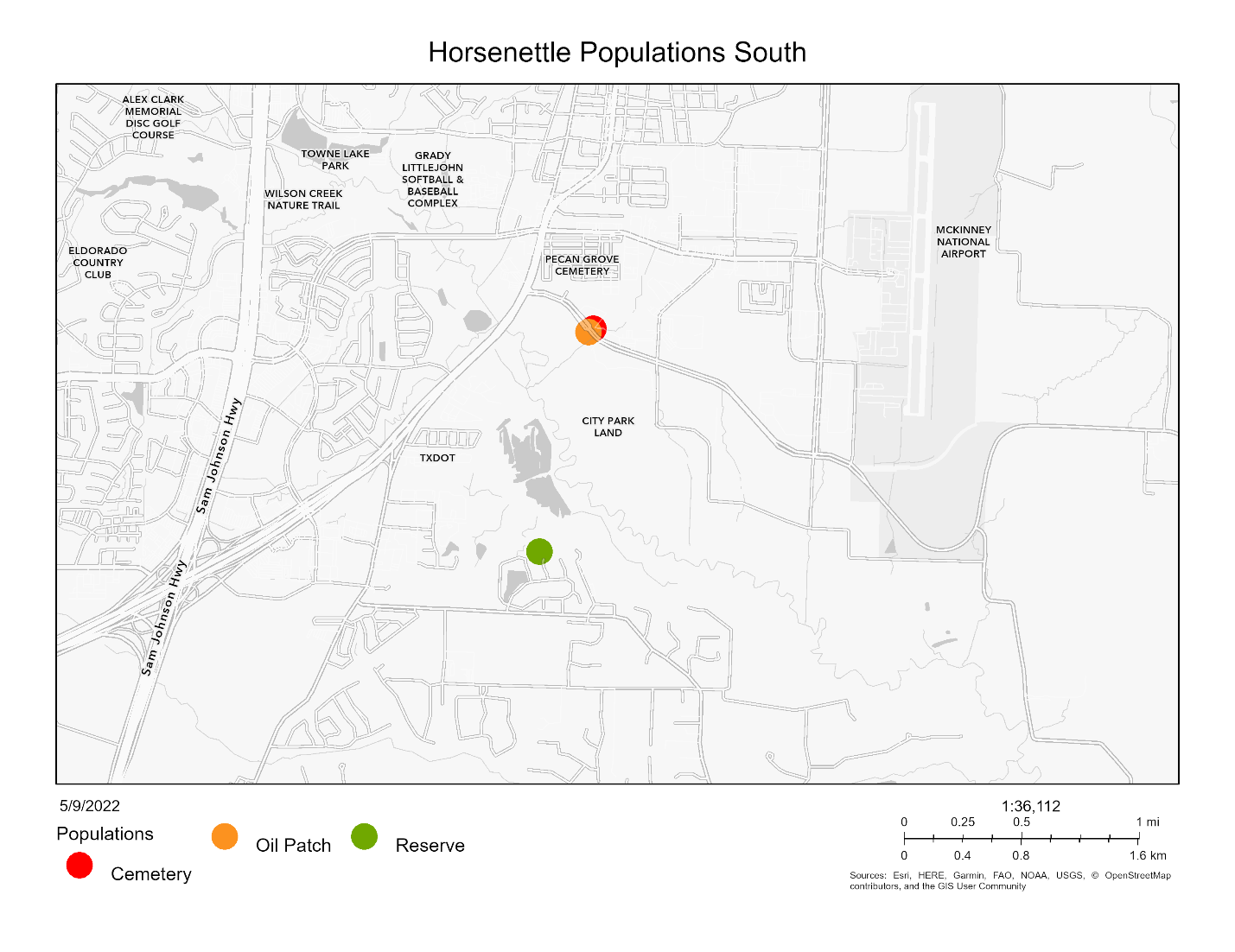
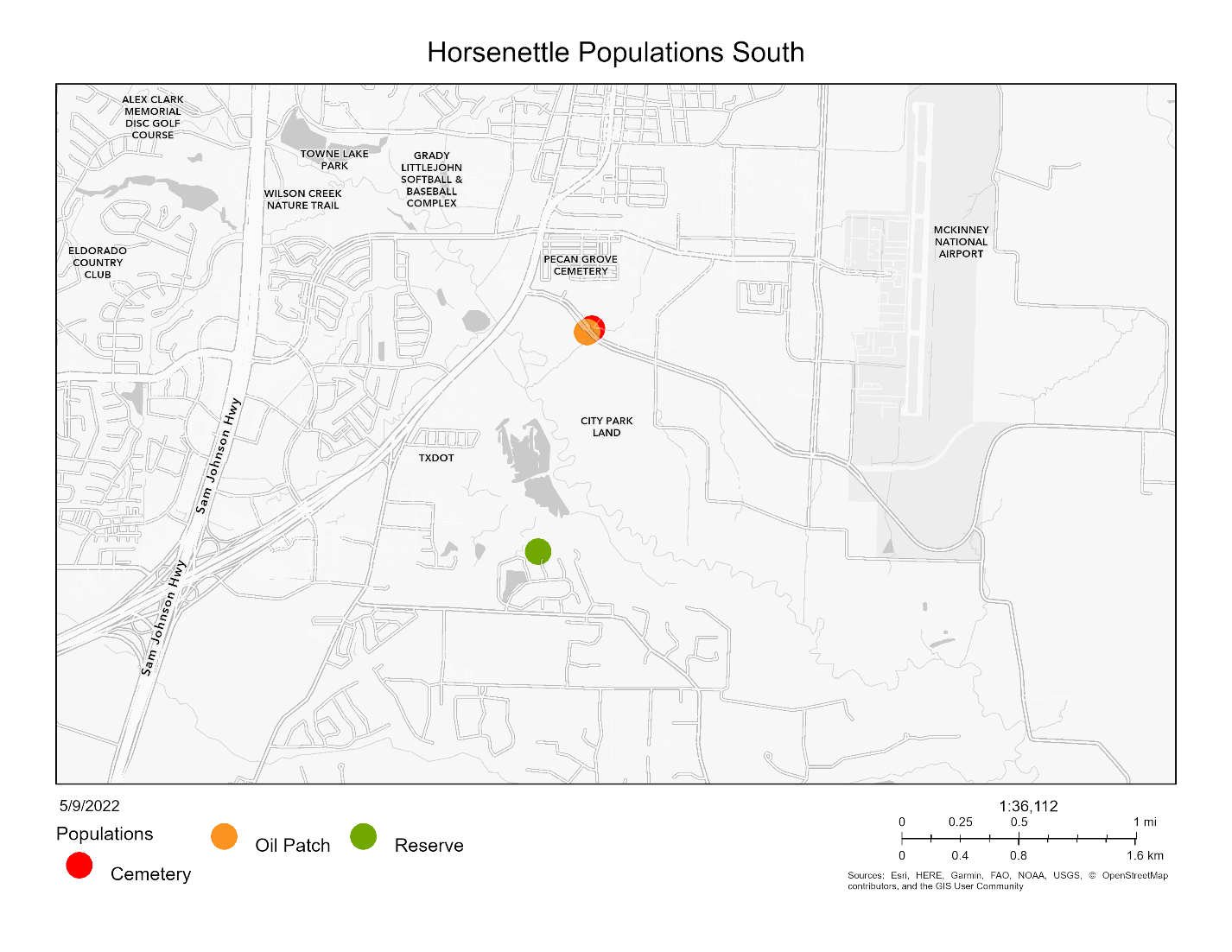
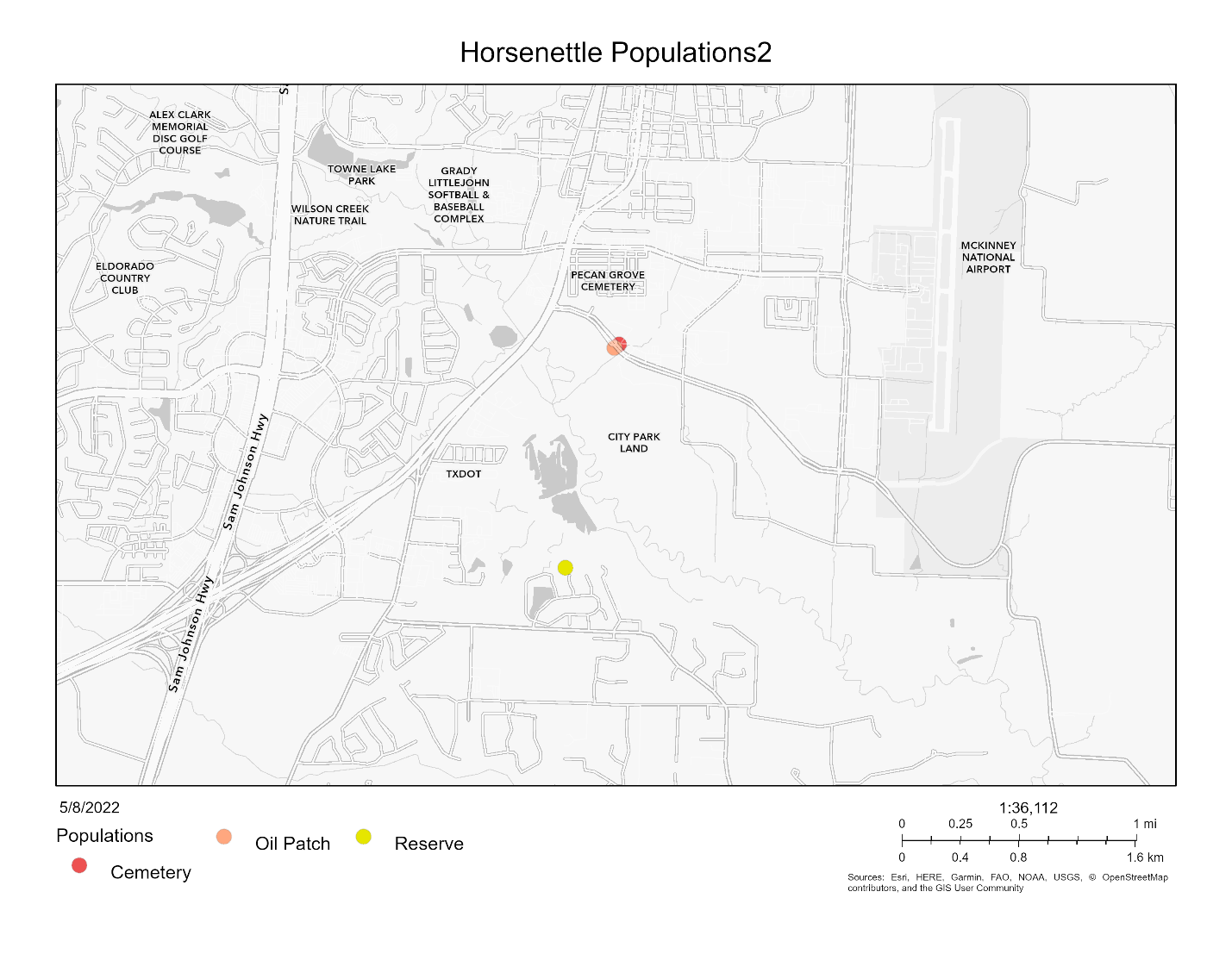
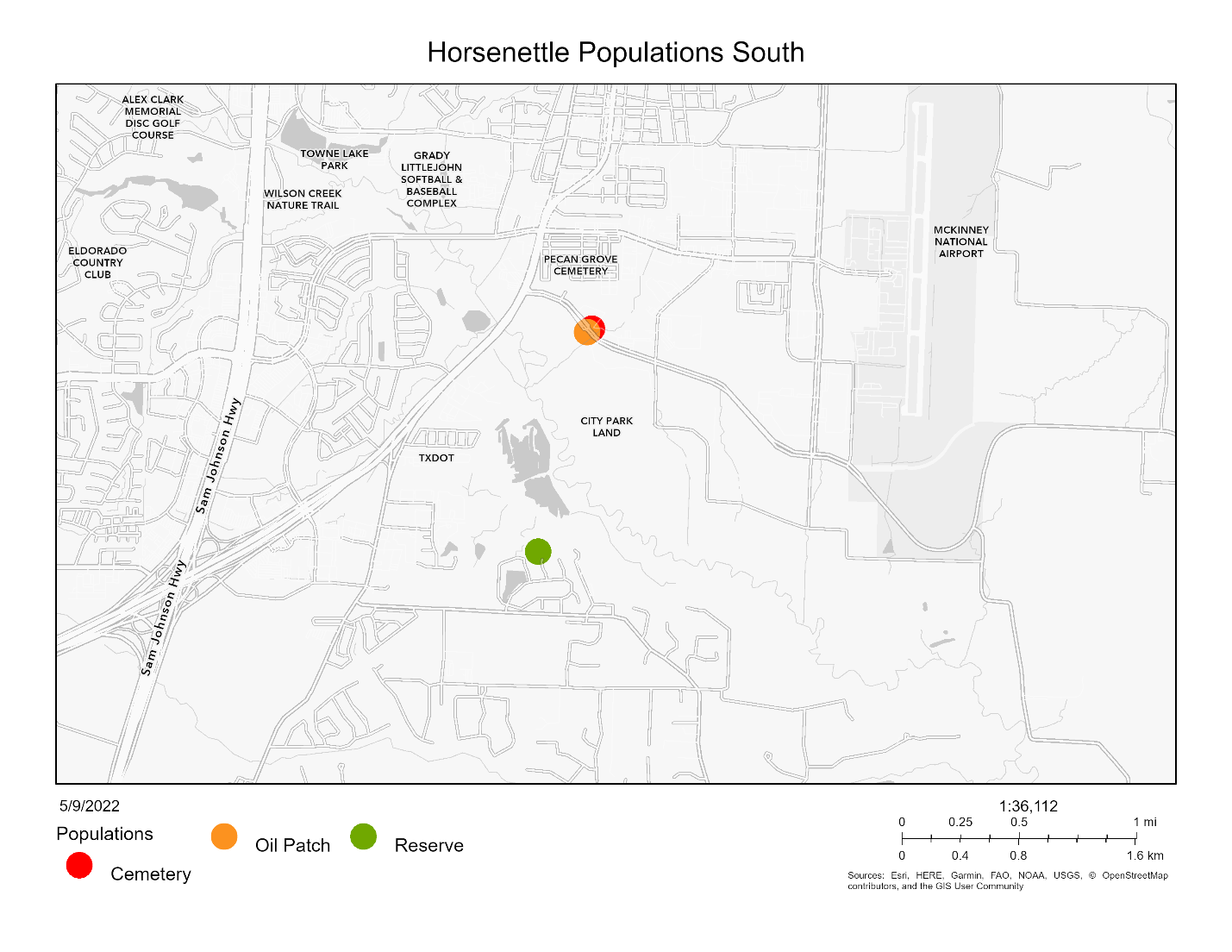


Figure 3. Populations in the southern region. Cemetery in red, Oil Patch in orange, and Reserve in green.

***Greenhouse Design***

After the dormancy period, sections of rhizome were equally cut to grow ramets (genetically identical) in cone-shaped containers in the greenhouse. In total, four ramets (blocks A, B, C, and D) were grown from each genet (genetically independent), separated temporally. We started 10 or 12 ramets each week (sub-block 1-20), randomly selected from the 52 genets. Of the 10 or 12 ramets planted each week, half were from the southern region and half were from the northern region. All ramets in block A were planted over five weeks before planting the ramets in block B and so on. The northern ramets were randomly assigned to either the left or right side of the respective southern pair within the tray that held the cone-shaped containers. The plants were fertilized regularly and transplanted to larger containers when they outgrew the small cone-shaped containers. Once the plants had leaves of a reasonable size, we began collecting sporophytic data from one sub-block each week. Gametophytic data were acquired when plants began flowering.

***Sporophytic: Chlorophyll Fluorescence Stability***

We used a chlorophyll meter to measure chlorophyll fluorescence stability (CHPL). Two intact leaves were removed from the middle of the plant and cut into half on either side of the midveins. One leaf was used for the heat treatment and the other was used for the cold treatment. One half was placed in the treatment and the other half was placed in the control of room temperature. The CHPL was measured for both halves before and after the temperature treatment.

The high temperature treatment was 60°C for 1 hour. The leaf halves in the cold treatment were subjected to 4°C for 1 hour followed by 1 hour in -18°C. The leaf halves were moved to room temperature for two hours prior to the second cold treatment measurement. Leaves in all treatments were kept in complete darkness.

The CHPL value was calculated as the inverse of the proportion of treatment final to treatment initial divided by the proportion of the control final to control initial.

***Sporophytic: Cell Membrane Stability***

We used a handheld conductivity meter to measure cell membrane stability after a temperature treatment and again after a maximum damage treatment. Two large, intact leaves were removed from the middle of the plant, washed and each hole punched into 20 rounds. One leaf was used for the high temperature treatment and the second leaf was used for the cold temperature treatment. Ten of the 20 leaf rounds were placed in a test tube for the temperature treatment and 10 were placed in a test tube for the control treatment.

Prior to the high temperature treatment, 10 mL of deionized water was added to the control and temperature treatment test tubes. The high temperature treatment test tubes were placed in a water bath at 55°C for 20 minutes, while the control test tubes were left at room temperature. Both treatments were moved to room temperature for 10 minutes prior to the first conductivity measurement.

The low temperature treatment test tubes were placed at 10°C for 24 hours followed by 24 hours at 4°C to acclimate the leaf rounds to cooler temperatures. The test tubes were then placed at -18°C for 1 hour. The control treatment test tubes remained at room temperature. 10 mL of deionized water were added to all test tubes in both treatment and were placed at room temperature for 1 hour prior to the first conductivity measurement.

All treatments groups were subjected to a maximum damage treatment after the first conductivity measurements. The test tubes were placed in a water bath at 98°C for 1 hour and then left to cool at room temperature for 15 minutes before the second conductivity measurement.

The cell membrane stability value used for data analysis was calculated as one minus the proportion of treatment final to treatment group maximum divided by one minus the proportion of control final to control group maximum conductivity.

***Sporophytic: Net Photosynthetic Rate***

We used a LI-6400 infrared gas analyzer with a red/blue light source to measure photosynthetic rate before and after the temperature treatment. The following settings were used for photosynthesis measurements: flow rate 500 μmol s-1, reference CO2 420 μmol CO2 mol-1, reference H2O 0 mmol H2O mol-1, ParIn\_μml 400 μmol m-2 s-1.

The high temperature treatment was 33°C and the low temperature treatment was 10°C. Each ramet was subjected to both treatments with a rest period of one week between the temperature treatments. Ramets A and C were subjected to the high temperature treatment first and ramets B and D were subjected to the low temperature treatment first. The proportion of the measurement after the treatment to before was used in analysis. Any values below zero and above one were omitted prior to analysis.

***Gametophytic: Pollen Variables***

Once a plant from the north and from the south flowered, we removed a mature flower from both plants. Since *Solanum carolinense* is buzz pollinated, a device crafted from a nose hair remover and a paper clip was used to mimic the vibrations needed to release pollen from the anther. Pollen from one flower was dispersed over five plates with a 3% Bacto-Agar based growth medium (sucrose, Ca(NO3)2, MgSO4, KNO3, H3BO3). The plates were each placed at one of the five temperature treatments (10°C, 20°C, 25°C, 30°C, 40°C) for 16 hours. After the temperature treatments, the plate was covered with a thin layer of ethanol to halt further pollen tube growth. Four pictures of each plate were taken using a microscope and ImageJ was used to collect pollen germination and pollen tube growth rate data.

Pollen germination was measured by counting the number of pollen grains that produced pollen tubes and the number of pollen grains that did not produce pollen tubes in the first picture taken. A pollen tube was had to be at least half the diameter of the pollen grain to be included in the count. We used the percent of pollen grains with tubes out of the total number of pollen grains for data analysis.

Pollen tube growth rate was determined by measuring the 10 longest pollen tubes in each of the 4 pictures, calculating the mean of the 20 longest out of the 40 measured, and dividing that length in millimeters by 16 hours. Pollen tubes were only included if they were completely visible in the picture.

***Data Analysis***

All data were analyzed in R (version 4.1.2). For each of the sporophytic variables, we used linear mixed effects models (LMM; function lmer) to determine if there were differences between north and south. Region was the fixed effect and block and genet nested in population were the random effects. We dropped the genet nested in population term for cell membrane stability and both random effects terms for hot net photosynthetic rate to avoid overfitting the model. Since the genet nested in population term was significant for some variables, we compared population and genets independently. Populations were compared using a linear mixed effects model (LMM; function lmer) with population as the fixed effect and block as the random effect. We used an analysis of variance model (stats; function aov) to determine if there were differences between genets for each of the sporophytic variables. Since there was a significant block effect in some of the variables, we compared plants from the north and south within block using a paired t-test (stats; function t.test). To determine if variation within the northern and southern regions differed, we used the bartlett’s test of homogeneity of variance (stats; function bartlett.test).

For the gametophytic variables, we fit temperature performance curves (rTCP: function nls.multstart) for each plant that flowered. We fit two genets, one from the north and one from the south, with all 25 temperature performance curves available in the rTCP packages and used AIC values to select the quadratic\_2008 and the weibull\_1995 models. We opted to use the quadratic\_2008 model because the temperature maximum values determined by the weibull\_1995 model were infinite for some of the plants. From the quadratic curves of all plants that flowered, we extracted the temperature minimum, temperature optimum, and temperature maximum values and used analysis of variance (stats; function aov) to determine if there were differences between region and genet.

We used correlation analysis (stats; function cor) to determine if there were any correlations between sporophytic and gametophytic variables. We did correlation analysis for all plants together and then the northern and southern plants separately. To further determine if there were relationships between the variables and whether those differed for region and population, we conducted principal component analysis (PCA) (stats; function prcomp). We used PCA on all the sporophytic variables and all gametophytic variables separately and then both sporophytic variables and gametophytic variables excluding photosynthesis. For the gametophytic and the photosynthesis data, we did not have full data sets and using both limited the sample size for PCA, therefore we opted to remove photosynthesis. We extracted the eigenvalues for the first three principal components for all three PCAs. The eigenvalues for each principal component were compared for the two regions using t-tests (stats; function t.test).

**Results**

***Sporophytic Variables***

*Cell Membrane Stability*

Cell membrane stability (CMS) was quantified using the ratio of a conductivity measurement after a temperature treatment to a conductivity measurement after a maximum damage treatment. An increased CMS ratio indicates higher tolerance of the temperature treatment. When *Solanum carolinense* plants from the north were compared to the south, we found no significant difference in the hot treatment, but there was a significant difference in the cold treatment (figure 1). We found a significant difference among genotypes in the hot treatment, but not in the cold treatment (figure 2).

Because we could not grow all the experimental plants at the same time due to lack of space, we made the above comparisons among regions and genotypes in five different temporal blocks over the course of the spring and summer. To avoid confounding treatments with temporal effects plants from different regions were paired with each other within blocks. When we tested for the presence of block effects, we found significant effects for both hot and cold CMS (figure 3). Plants grown at different times in the greenhouse had different CMS ratios. We started growing the plants in the winter and early spring and outside temperatures gradually rose during that time (figure 4). Acclimation to higher temperatures later in the year could account for the block differences observed. The most even temperatures were in the winter, when the earliest ramet was planted. When plants from the north and south were compared for hot CMS (HCMS) in just ramet A in a paired t-test, there was a significant difference between the regions (figure 3). Northern plants had a higher CMS in extreme heat than those from the south. For cold CMS (CCMS), there was a significant difference between regions for blocks B and C (figure 3). In both cases, southern plants were more tolerant of the cold temperatures than northern plants.

*Chlorophyll fluorescence stability*

Chlorophyll fluorescence was measured before and after either a heat stress or cold stress and the inverse ratio of the measurements was used as a proxy for temperature tolerance. As the chlorophyll ratio increases, the individual sporophyte is more tolerant of the temperature treatment. There was a significant difference between plants originating in the north and south for both the hot and cold treatments (figure 1). Northern plants were more tolerant than southern plants in both treatments regardless of block. We found a significant difference between individual genotypes for the cold treatment, but not for the hot treatment (figure 2). The two regions also differed in variation for hot CHPL. Northern plants had significantly more variation in the hot treatment than southern plants (Bartlett’s test p-value = 1.68E-4) (figure 5). No other variable showed a difference in variance between the two regions.

*Net Photosynthetic Rate*

We used net photosynthetic rate after thermal stress as a physiological indicator of temperature tolerance. Both previous variables mentioned could directly influence temperature tolerance through their involvement in photosynthesis. Net photosynthetic rate (PS) was the proportion of the net photosynthetic rate after the treatment (heat and cold) to the net photosynthetic rate before the treatment. Increased PS indicates higher temperature tolerance of the thermal stress. For both the cold and hot treatments, there was no significant difference between north and south (figure 1). There were no significant differences between blocks and genotypes for both the hot and cold treatments.

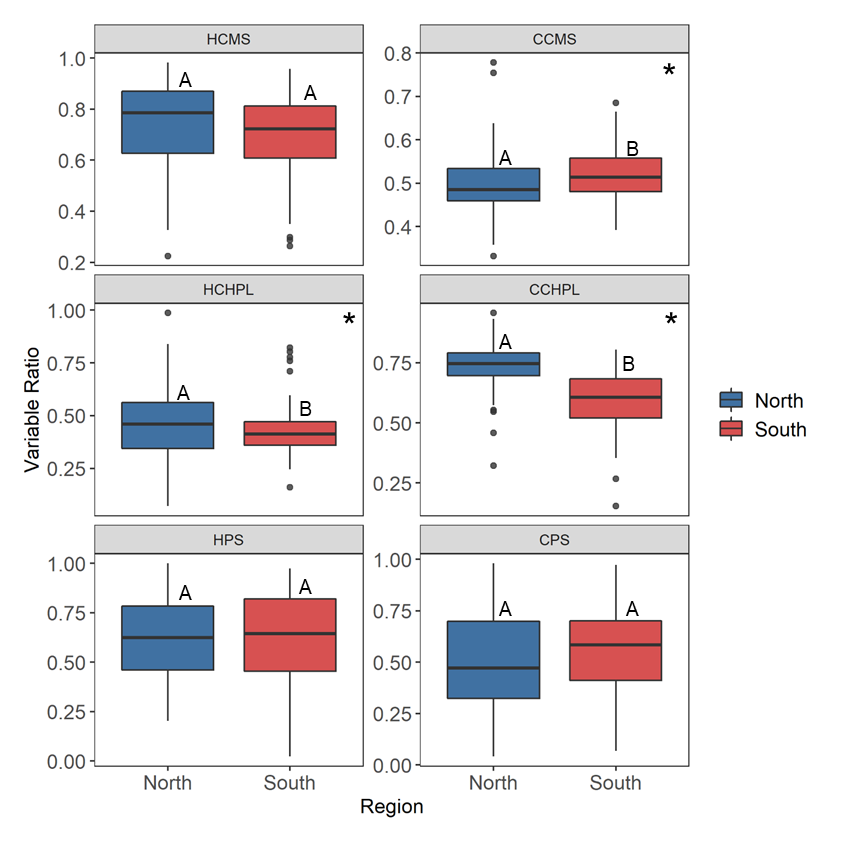
Figure 1. Regional differences for temperature tolerance traits including hot and cold cell membrane stability (HCMS, CCMS), hot and cold chlorophyll fluorescence stability (HCHPL, CCHPL), hot and cold net photosynthetic rate (HPS, CPS). Center line of boxplot is the median value for the region. The letters represent statistically significant differences between regions. Variables with significant differences denoted with asterisks: CCMS (F1,50 = 7.792, p = 0.006), HCHPL (F1,51 = 4.334, p = 0.043), and CCHPL (F1,50 = 64.652, p = 1.6e-10).

Table 1. Results from the mixed linear model for the difference in region (north vs south) and the one-way analysis of variance results for the difference between individual genets. Red font color highlights observed outcomes when the result was different from the expected pattern. Asterisk indicates analysis with one outlier removed determined using Grubbs test for one outlier.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **Region** | | | **Genet** | |
|  | **Variable** | **Expected** | **Observed** | **p-value** | **Observed** | **p-value** |
| **Sporophyte** | Cell Membrane Stability (Heat) | S > N | - | 0.06102 | **Yes** | **0.013** |
| Cell Membrane Stability (Cold) | N > S | **S > N** | **0.0117** | No | 0.886 |
| Chlorophyll Fluorescence (Heat) | S > N | **N > S** | **0.0405** | No | 0.38 |
| Chlorophyll Fluorescence (Cold) | N > S | N > S | **9.96E-11** | **Yes** | **1.05E-07** |
| Photosynthetic Rate (Heat) | S > N | - | 0.997 | No | 0.127 |
| Photosynthetic Rate (Cold) | N > S | - | 0.77 | No | 0.883 |
| **Gametophyte** | Pollen Germination (Tmax) | S > N | **N > S** | **0.00037** | **Yes** | **0.0251** |
| Pollen Germination (Topt) | S > N | **N > S** | **0.000685** | **Yes** | **0.0351** |
| Pollen Germination (Tmin) | S > N | - | 0.331 | **Yes** | **\*0.0135** |
| Pollen Tube Growth Rate (Tmax) | S > N | - | 0.568 | No | 0.418 |
| Pollen Tube Growth Rate (Topt) | S > N | - | 0.77 | No | 0.608 |
| Pollen Tube Growth Rate (Tmin) | S > N | - | 0.683 | No | 0.496 |

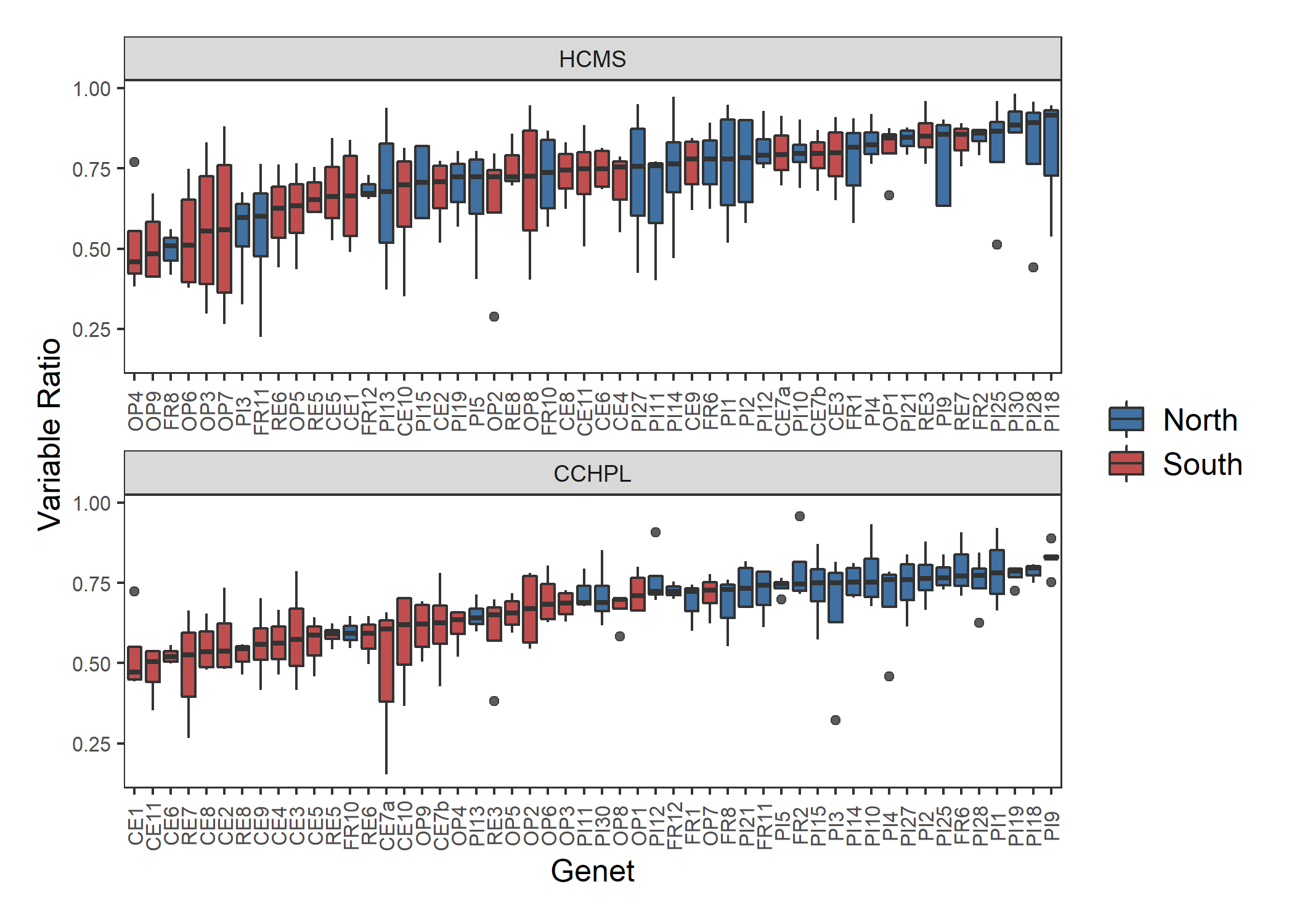


Figure 2. Genotype differences for temperature tolerance traits including hot cell membrane stability (HCMS) and cold chlorophyll fluorescence stability (CCHPL). Genets ordered by increasing ratio for each variable. Center line in boxplot is the median of the measurements taken for the ramets of one genet. There is a significant difference among the genets for HCMS (F = 1.5, p = 0.029) and CCHPL (F = 3.341, p = 6.1e-9). Plots of genet effect for other variables in appendix.

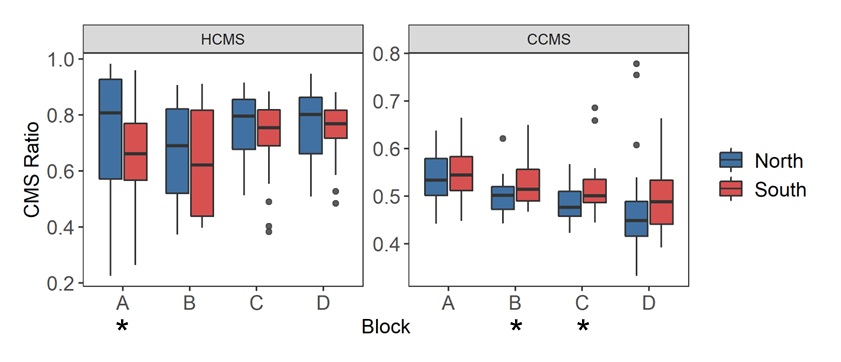


Figure 3. Cell membrane stability across temporally independent blocks and colored by region. The center line of the boxplot is the median of the measurements taken for each region within a ramet. There is a significant difference between blocks for hot cell membrane stability (HCMS, p = 0.0297) and cold cell membrane stability (CCMS, p = 7.30e-05). Astrisks indicate a significant difference between regions from a paired t-test of regions for each block independently. There was a significant difference between regions for HCMS block A (t = -2.910, p = 0.015), CMS block B (t = 2.190, p = 0.040), and CMS block C (t = 2.073, p = 0.049). Results from paired t-tests between blocks for each variable located in the appendix.

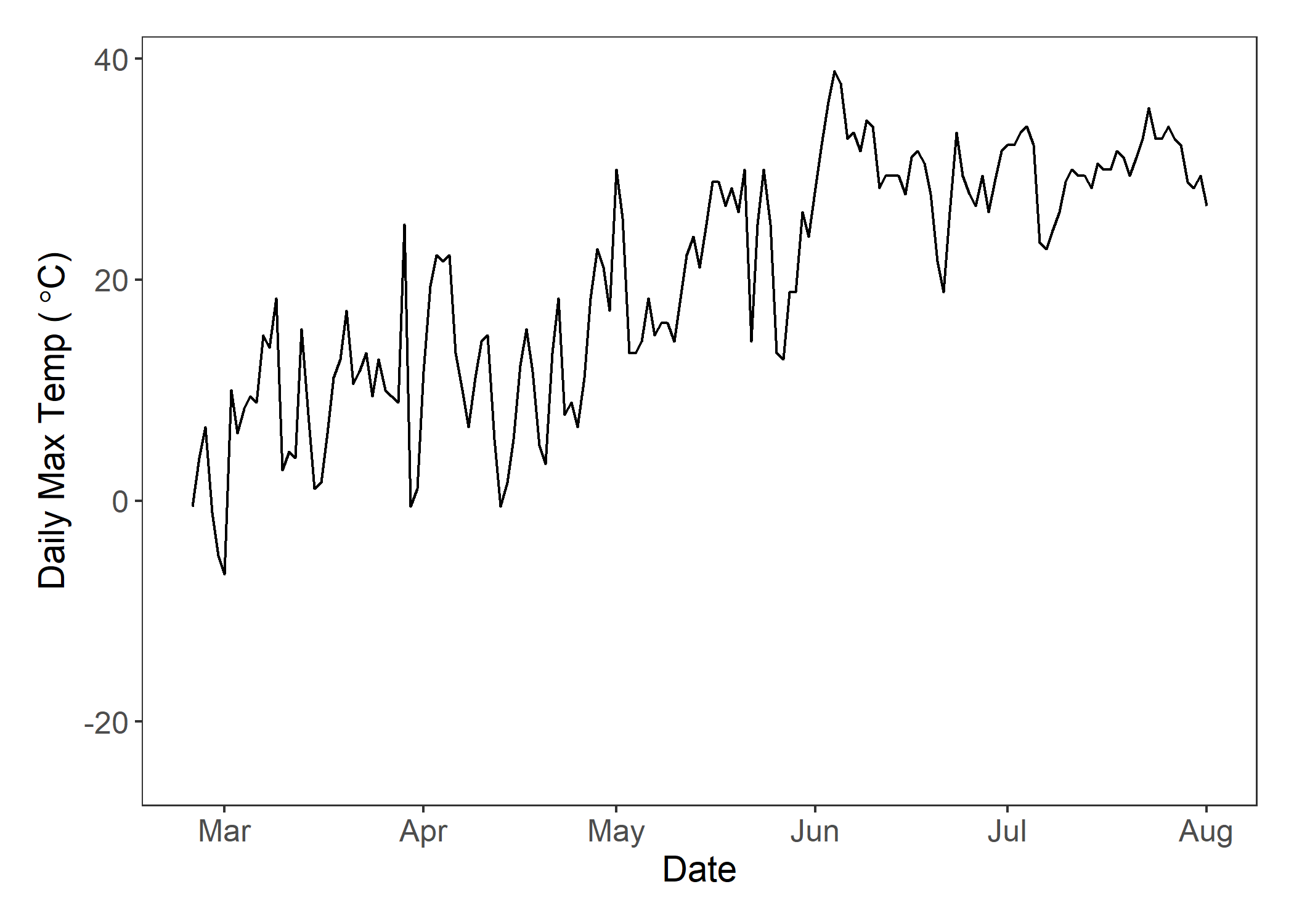


Figure 4. Daily max temperature for spring and summer from the NOAA station at the Hector International Airport, Fargo, ND.

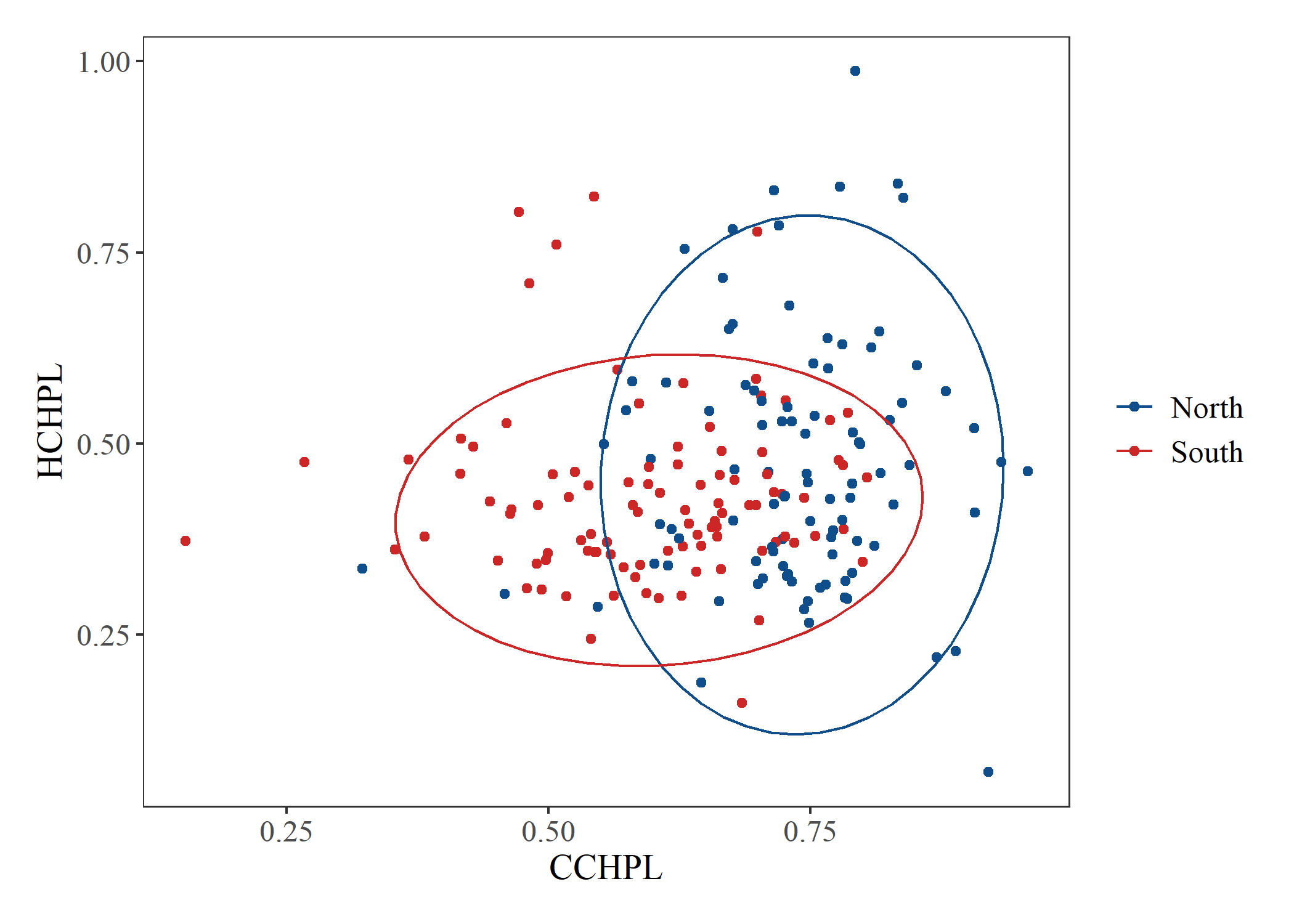


Figure 5. Hot chlorophyll fluorescence stability (HCHPL) vs cold chlorophyll fluorescence stability (CCHPL) for plants from the north and south. Ellipse indicating 95% confidence interval for multivariate T distribution. Results from Bartlett’s test for heterogeneity of variance between regions for all variable located in the appendix.

***Gametophytic Variables***

*Pollen Germination*

We fit quadratic curves to temperature performance profiles of each genet for pollen germination at five temperatures (figure 7). From the quadratic fit, we calculated the minimum (Tmin), maximum (Tmax), and optimal (Topt) temperature of pollen germination for each individual. There was a significant difference between regions for Tmax and Topt (figure 8). Genets from the north germinated more readily at high temperatures than genets from the south. There was no significant difference between the two regions for Tmin. The genets were significantly different from one another for Tmin, Tmax, and Topt (figure 9). One outlier was identified using the Grubbs test for one outlier (outliers; function grubbs.test) and subsequently dropped from the analysis.

*Pollen Tube Growth Rate*

The pollen tube growth rates for each individual were also fit with a quadratic curve to estimate the Tmin, Tmax, and Topt. There were no significant differences between plants from the north and south for each of the three variables (Appendix). There were also no significant differences among genets (Appendix).

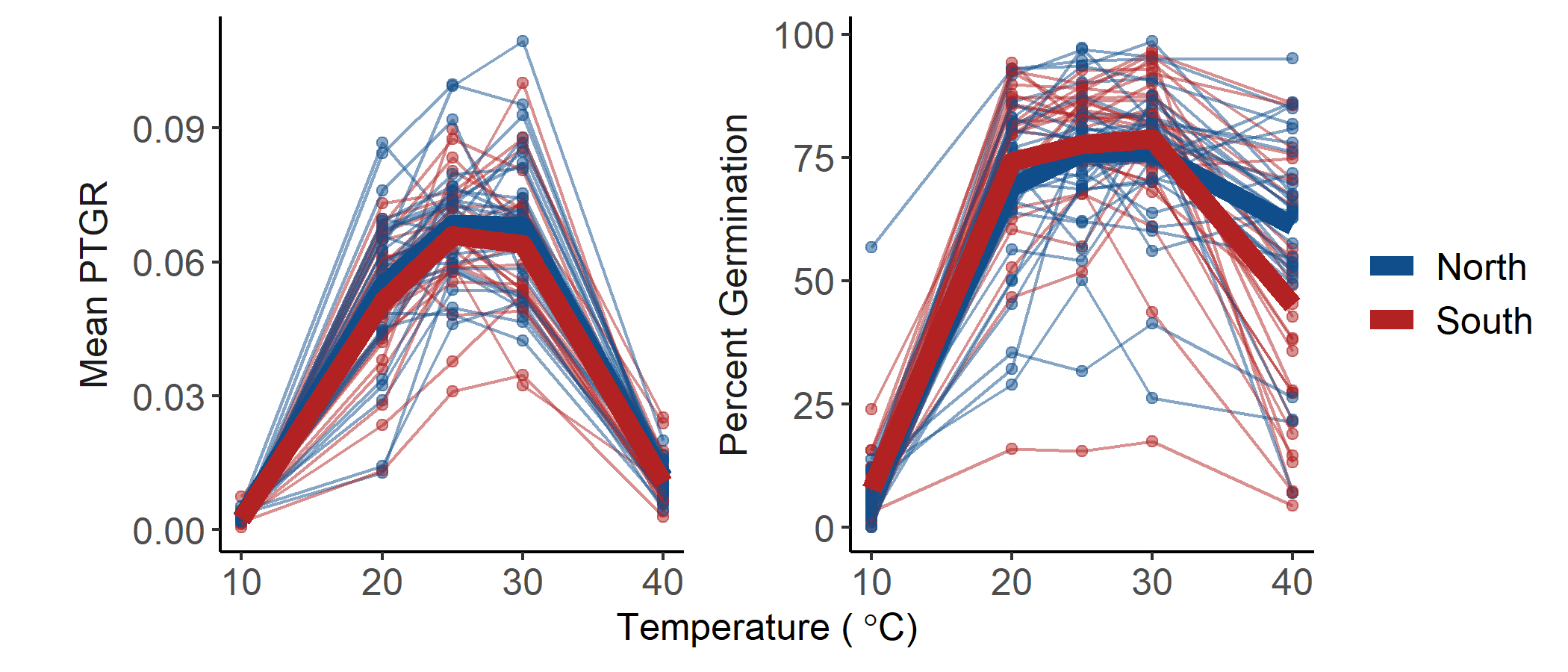


Figure 6. Percent germination and mean pollen tube growth rate (PTGR) for *Solanum carolinense* pollen grains from the north (blue) and south (red) across a temperature gradient (10°C, 20°C, 25°C, 30°C, 40°C). Thin lines and points represent each individual plant that flowered. Thick lines indicate the mean value for the region at each temperature.

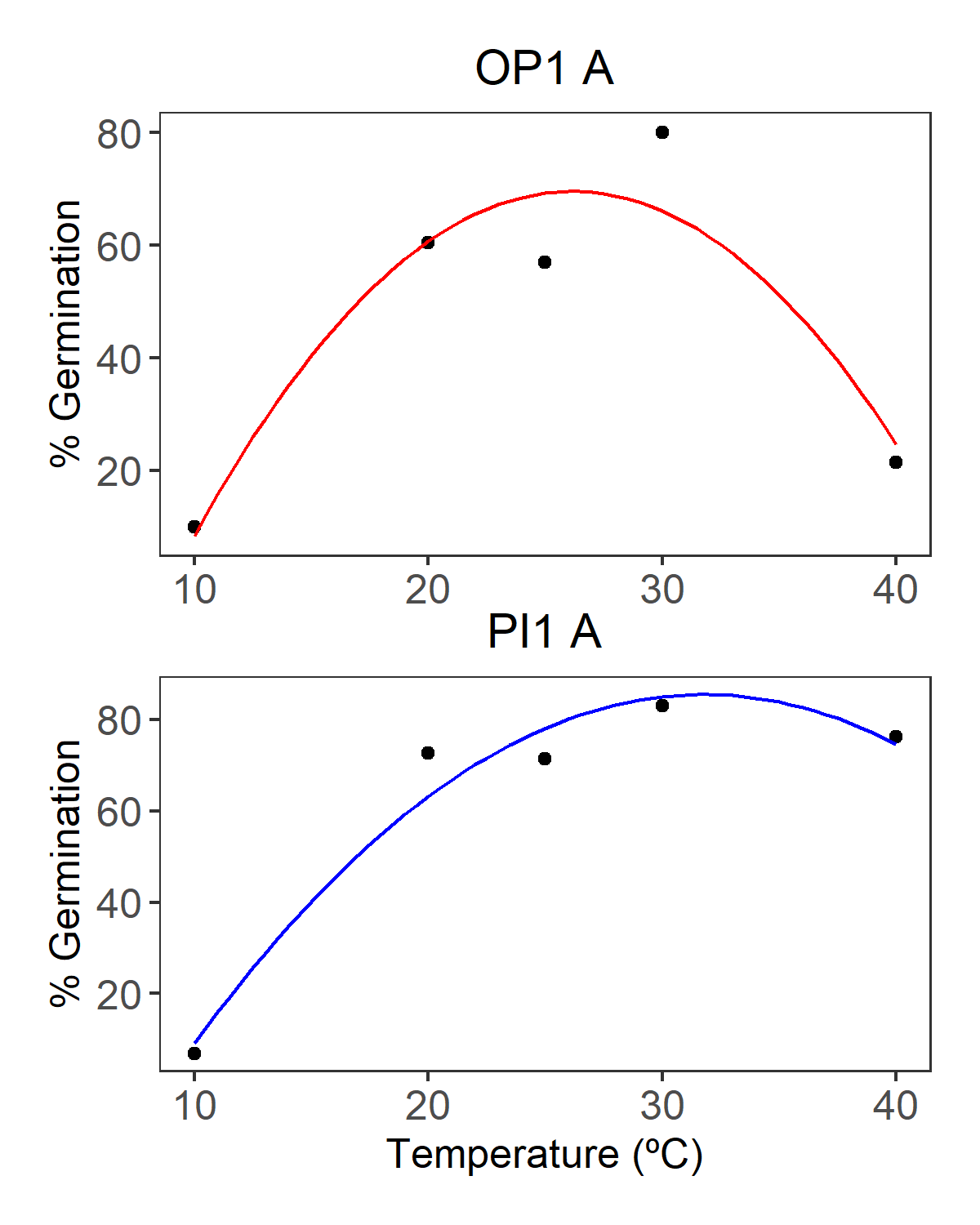


Figure 7. Examples of quadratic fit curve for pollen germination of one genet from the southern region (OP1 A, red) and one genet from the northern region (PI1 A, blue).



A

B

\*

A

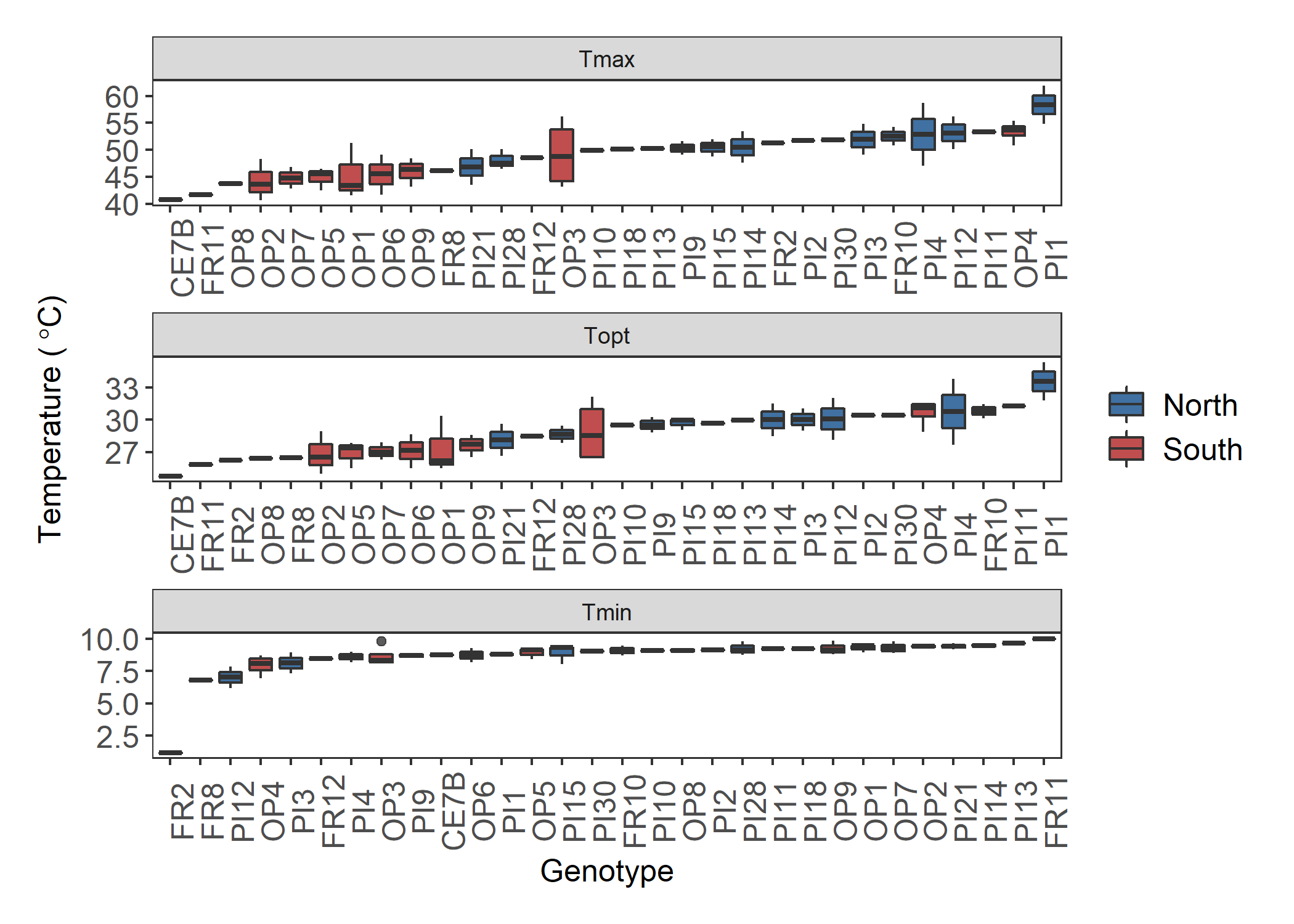
B

\*

A

A

Figure 8. Estimates for the maximum (Tmax), optimal (Topt), and minimum (Tmin) germination temperature extracted from quadratic fits of the germination data for each individual. Asterisks and different letters indicate significant differences. There is a significant difference between regions for Tmax (F = 14.28, p = 3.7E-4) and Topt (F = 12.85, p = 6.85E-4).



\*

Figure 9. Boxplots of the maximum (Tmax), optimal (Topt), and minimum (Tmin) pollen germination temperatures by genet. There is a significant difference between the genets for Tmax (F = 2.064, p = 0.025), Topt (F = 1.952, p = 0.035), and Tmin (F = 2.284, p = 0.0135). Asterisk indicates the outlier removed for analysis. Plots of genet effect for other variables in appendix.

Chart, bubble chart

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Figure 10. Correlation matrix of northern plants. Sporophytic (dark gray font) and gametophytic variables (black font) with significant Pearson’s correlations for all study plants. Blue colors indicate positive correlations and red colors indicate negative correlations.

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Figure 11. Correlation matrix of southern plants. Sporophytic (dark gray font) and gametophytic variables (black font) with significant Pearson’s correlations are included. Blue colors indicate positive correlations and red colors indicate negative correlations.

***Correlations***

We used correlation analysis analysis to identify relationships between hot and cold tolerance for the sporophytic and gametophytic variables. Pearson’s correlations were determined for all pairings of variables. When all plants were included, there were no significant correlations between the gametophytic and sporophytic variables (Appendix). Of the sporophytic variables, only three correlations were significant (correlation plots located in appendix). HCMS was weekly correlated with HPS (correlation = 0.19), CPS (correlation = 0.2), and CCHPL (correlation = 0.15) There were three significant correlation coefficients between the gametophytic variables. Maximum and minimum pollen tube growth rate were positively correlated (correlation = 0.45). Maximum and minimum pollen germination were negatively correlated (correlation = -0.33). Maximum pollen tube growth rate and maximum pollen germination were positively correlated (correlation = 0.3).

When the correlation analysis was performed for the regions separately, there were different results. For the northern plants, there was only one significant correlation between HPTGR and CPTGR (Pearson’s correlation 0.59; figure 10). The southern plants had several significant correlations (figure 11). For the sporophyte, there was one correlation between HCMS and HPS (correlation = 0.47). For the gametophyte, there were correlations between cold germination (Cgerm) and hot germination (Hgerm) (correlation = -0.63) and between hot PTGR (HPTGR) and cold PTGR (CPTGR) (correlation = 0.51). In the southern plants, were correlations between the gametophytic and sporophytic life stages. Cgerm was negatively related to CCMS (correlation = -0.37) and positively related to HPS (correlation = 0.63). Hgerm was also related to HPS (correlation = -0.57), but negatively. HCHPL and CPTGR were negatively correlated (correlation = -0.39), while CCHPL is negatively related to HPTGR (correlation = -0.41).

***Principal Component Analysis***

We conducted principal component analysis to further explore relationships among all variables and the sporophytic and gametophytic variables separately. For the full PCA, we included all gametophytic and sporophytic variables, except HPS and CPS. There were missing values for both photosynthesis and the gametophytic variables, due to plants dying or not flowering. To increase the sample size for the full PCA and incorporate both the sporophyte and gametophyte, we excluded the photosynthesis variables. The first three principal components accounted for 57% of the variation (full PCA plots and loadings located in the appendix). There was little divergence between regions. When the eigenvalues of the principal components were compared between region, PC2 was the only principal component with a significant difference between the regions (t58 = -2.69, p = 0.0092). CHPL loads primarily on PC2 and is likely driving the divergence between northern and southern plants. This pattern also occurs in the PCA for only sporophytic variables.

*Sporophytic PCA*

In the sporophytic PCA, the first three principal components explained 60% of the variation. HCMS and HPS primarily loaded on PC1 (Table 2, figure 12). PC2 was mostly influenced by CCHPL and PC3 by HCHPL. CCMS and CPS loaded evenly on two or more of the three axes. There was a significant difference between the regions for the eigenvalues extracted from both PC2 (t78 = -5.09, p = 2.39e-06) and PC3 (t101 = 2.38, p = 0.019). The divergence in PC2 can be explained by the opposite responses we observed for CCMS and both chlorophyll fluorescence treatments. Northern plants have a higher chlorophyll fluorescence ratio for both treatments, while southern plants had less cell membrane damage in the cold treatment. PC1 did divide HCMS and CCMS, suggesting an antagonistic relationship between the two variables, though there was no correlation between the two that was statistically significant. Hot and cold treatment variables were also divided on PC3. HPS and HCHPL were opposite in direction to CPS and CCHPL.

*Gametophytic PCA*

In the gametophytic PCA, the first three components explained 92.5% of the variance. Pollen germination variables divided the northern and southern plants. Tmax and Topt loaded evenly in the opposite direction of Tmin for both PC1 and PC2 (Table 3, figure 13). There was a significant difference between north and south for the eigenvalues extracted from PC2 (t46 = -3.17, p = 0.0025). PTGR variables loaded evenly on the first two principal components and in the same direction.

Chart

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Figure 12. Plots of the results of principal component analysis for the sporophytic variables. A) PC1 and PC2, B) PC2 and PC3, C) PC1 and PC3. Ellipsoid indicating 95% confidence interval. PC1 explains 22.38% of the variance, PC2 explains 21.55% of the variance, and PC3 explains 16.79% of the variance. Tables with principal component importance for PC1 through PC6 in the Appendix.

Table. Results from principal component analysis with sporophytic variables. Loadings for each of the variables on the principal components

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **PC6** |
| **HCMS** | 0.613999 | 0.02998 | 0.344975 | 0.147492 | 0.573797 | 0.390002 |
| **CCMS** | -0.35207 | 0.435008 | 0.204072 | -0.57534 | 0.520509 | -0.20789 |
| **HCHPL** | 0.284794 | -0.36797 | -0.33836 | -0.75803 | -0.08744 | 0.294536 |
| **CCHPL** | 0.117118 | -0.57752 | 0.579996 | -0.14114 | -0.03843 | -0.5431 |
| **HPS** | 0.577968 | 0.302596 | -0.40867 | 0.000171 | 0.044159 | -0.63673 |
| **CPS** | 0.264909 | 0.499375 | 0.470594 | -0.22955 | -0.6235 | 0.13244 |

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Figure 13. Plot of the results of principal component analysis of the gametophytic variables. PC1 describes 48% of the variation and PC2 explains 27%. A table of importance of principle components 1 through 6 is in the Appendix.

Table 3. Results from principal component analysis with gametophytic variables. Loadings for each of the variables on the principal components

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **PC6** |
| **Germ.Tmin** | -0.01334 | 0.405643 | 0.812217 | 0.376238 | -0.00058 | -0.18446 |
| **Germ.Topt** | 0.418665 | -0.45436 | 0.390064 | -0.00262 | 0.001723 | 0.682727 |
| **Germ.Tmax** | 0.407763 | -0.5446 | 0.164764 | -0.10069 | -0.00179 | -0.707 |
| **PTGR.Tmin** | 0.367661 | 0.452838 | 0.127813 | -0.75119 | -0.28131 | 0.00071 |
| **PTGR.Topt** | 0.523981 | 0.308129 | -0.20261 | 0.123985 | 0.757676 | -0.00193 |
| **PTGR.Tmax** | 0.498538 | 0.180049 | -0.3219 | 0.518304 | -0.58888 | 0.001496 |

**Discussion**

***Regional Differences***

If *Solanum carolinense* has locally adapted to the respective temperature regimes in TX and MN, we would expect that plants from the north would be more tolerant of cold temperatures and plants from the south would be more tolerant of hot temperatures. Rather than a clear-cut difference between north and south for hot and cold treatments, there were mixed results that support divergence between regions in ways we hadn’t anticipated.

*Sporophyte*

Plants from the south had a more stable cell membranes when exposed to an extreme cold treatment. Cold stress reduces the fluidity of the cell membrane and produces ROS that have the potential to oxidize lipids and damage the membrane (Valitova et al., 2019). The incorporation of sterols in membranes can maintain fluidity and expand temperature range for plants (Dufourc, 2008a, 2008b; Valitova et al., 2019). Conversely, saturated fatty acids can be incorporated in the cell membrane to reduce fluidity and are often associated heat tolerance (Knight & Ackerly, 2001; Zhu et al., 2018). There was no significant difference between region for HCMS for all study plants together, but there was a significant difference for plants in block A. Temperatures in the greenhouse progressively rose throughout the spring and summer leading to a block effect in both the hot and cold treatments of CMS. In block A for HCMS measurements, northern plants had a higher HCMS, but this difference degraded in the later blocks during the times when temperatures were higher. Southern plants may have the capacity to induce heat tolerance as they acclimate to warmer conditions. Block A may be the best representative measurement of baseline heat tolerance for HCMS, but the later blocks suggest that CMS is a plastic trait. The median of HCMS generally increased across blocks, while the median of CCMS decreased. While there was no significant correlation between CCMS and HCMS, these results suggest that HCMS and CCMS are inversely related. Our PCA results also showed an antagonistic relationship between the CMS variables as they were loaded in opposite directions for PC1 and PC2 in the sporophytic PCA.

HCMS was an important variable in the correlation analysis that included all plants. HCMS was weakly and positively correlated with CCHPL, CPS, and HPS. Only one of these correlations was significant when the regions were analyzed separately. HCMS was positively correlated with HPS. These results indicate that membrane structural integrity is related to photosynthetic rate in heat. Since the light reaction does occur in the thylakoid membrane within chloroplasts, the rigidity of cell membranes in heat may directly affect efficiency of energy absorption and electron transport.

There was no significant difference between northern and southern plants for net photosynthetic rate in both the hot and cold treatments. Net photosynthesis was the only sporophytic variable where the whole plant was placed in a temperature treatment and leaves were measured on the plant. The plant may compensate for temperature stress through physiological mechanisms, such as increasing transpiration. Therefore, the temperature treatments may not have stressed the plants to the extent that temperature tolerance for the northern and southern plants was distinguishable.

Plants from the north had more stable chlorophyll fluorescence in both the hot and cold treatments. Stable chlorophyll fluorescence in heat and cold for northern plants may be explained by northern plants experiencing a larger range of temperatures. Between 2018 and 2021, temperatures during the growing season (March to September) in Houston County, MN ranged from -28°C to 34°C (62°C difference), while in Collin County, TX they ranged from -7°C to 42°C (49°C difference). Since the temperate conditions of Minnesota are more variable, populations in the north may have evolved to have higher levels of temporary phenotypic plasticity, at least when it comes to chloroplast and chlorophyll stability than those in the south. Furthermore, northern plants also had significantly more variation in HCHPL than southern plants. This may suggest that there is stabilizing selection occurring in the southern region for heat tolerance in chlorophyll stability. Less variation in HCHPL in the south may contribute to the counter-gradient results we attained. If northern plants experience less heat stress selection and have greater variation, then there may be more potential to have individuals with high HCHPL.

*Gametophyte*

To test gametophytic temperature tolerance in *Solanum carolinense*, we measured pollen performance variables over a temperature gradient. The variables of interest were pollen germination and pollen tube growth rate, both of which directly impact the capacity of a pollen grain to compete with other pollen and fertilize an ovule within a flower.

Pollen germination was higher in pollen grains from the northern plants than those in the south for both Tmax and Topt. This means that pollen from the north have a higher propensity to produce pollen tubes at high temperatures than their southern counterparts. The distinct difference between north and south suggests that there is sensitivity to high temperatures and likely an adaptive response occurring in the populations of the south. Rutley et al. (2022) proposed the two-baskets model categorizing pollen, which states that there are high-ROS (reactive oxygen species) and low-ROS subpopulations of pollen within anthers of flowering species. The low-ROS pollen have a lower metabolic rate than high-ROS pollen due to partial dehydration during development. The two subpopulations of pollen are adaptive as they allow for asynchrony in pollen germination, permitting some pollen to remain dormant in a stressful environment and grow pollen tubes later in more favorable conditions. Keller et al. (2018) found that *Solanum lycopersicum* (tomato) pollen had two responses during heat stress – direct and delayed translation. Luria et al. (2019) later showed that *Solanum lycopersicum* has pollen that fall in the low-ROS and high-ROS groups, supporting the two-basket model in a species closely related to *Solanum carolinense*. We hypothesis that *Solanum carolinense* populations in the south have higher proportions of low-ROS to high-ROS pollen grains than those in the north due to stronger selection from increased exposure to extreme heat in the south. Low-ROS pollen that remains dormant would not be adaptive in northern populations, with little exposure to high temperature stress.

There was a significant negative correlation between Tmax and Tmin germination. This correlation was driven by plants from the south and supports the two-basket model. The negative correlation means that plants with pollen that germinate readily at high temperatures also germinate at low temperatures, while those that have a lower Tmax have a higher Tmin. Plants with a higher proportion of high-ROS pollen would germinate in any condition (extreme heat and cold stress). Plants with a higher proportion of low-ROS pollen would not germinate as freely during stressful conditions. Since plants of the south have likely evolved to have the dual pollen types, there may be more variation in pollen activity driving this correlation. Southern plants also had a correlation between Tmax gemination and Tmax PTGR, meaning that if plants have pollen that germinate at higher temperatures, they also have pollen tubes that grow faster at high temperatures. PTGR is likely influenced by metabolic rate, which is increased in high-ROS pollen. There was a positive correlation between Tmin and Tmax PTGR in the north. This pattern indicates that pollen tubes either grow fast at high temperatures or low temperatures, but not both. When north and south were combined, there were positive correlations between germination and PTGR Tmin and between germination and PTGR Tmax, indicating that the response to temperature for gemination and pollen tube growth are related.

There was no significant difference between northern and southern populations for Tmin. Pollen of *Solanum carolinense* may be constrained by a lower temperature limit for the physiological processes necessary for pollen tube growth. Pollen tube growth rate also remained constant across regions and genets for the five temperatures. Since pollen tube growth rate is constrained by the physiological processes involved in cell division, there is likely little variation upon which selection can act.

***Inter-generational adaptations***

Tanksley et al. (1981) first described the correlation between selection in the gametophyte and sporophyte when they found a correlation between allozymes expressed in both stages. Based on their findings and several studies that followed (Hedhly et al., 2005; Pedersen et al., 1987; Poudyal et al., 2019; Willing & Mascarenhas, 1984), including studies on temperature tolerance (Hedhly et al., 2005; Poudyal et al., 2019), we hypothesized that there would be a correlation between temperature tolerance in the sporophyte and the gametophyte. There were no significant correlations between any of the gametophytic and sporophytic variables when northern and southern plants were both included in the correlation analysis. However, independently, southern plants did have strong relationships between gametophytic and sporophytic traits.

In the southern plants, most correlations between the sporophyte and gametophyte were negative but, one was positive. Cold germination was positively correlated to HPS. The positive correlation indicates that as the minimum temperature of pollen germination increases, net photosynthetic rate is maintained at higher temperatures. On the other hand, hot gemination was negatively correlated to HPS, meaning that as the maximum temperature of germination increases, net photosynthetic rate decreases in heat. Because two correlations involve germination and photosynthesis in southern plants, it is possible that pollen type determines photosynthetic resilience in heat. HPS was higher in plants with pollen that germinated in increased minimum temperatures and lower maximum temperatures, characteristics which are consistent with low-ROS pollen.

Chlorophyll fluorescence stability and pollen tube growth rate were also related to one another. CPTGR was negatively correlated with HCHPL and HPTGR was negatively correlated with CCHPL. These relationships suggest that cold tolerance and heat tolerance are antagonistic across stages. However, there are no positive correlations between the same variables and their counterpart in the same temperature treatment. Regardless, PTGR and chlorophyll fluorescence may both incorporate similar molecular responses to temperature in heat stress and a separate response for cold stress.

The last significant inter-generational correlation is between CCMS and cold germination. There was a negative relationship between the two, indicating that plants with decreased minimum temperature of pollen germination also had more stable cell membranes in cold stress. Often cold stress is mitigated at the cellular level by maintaining membrane fluidity. Cell membrane fluidity would also be important for pollen tube growth in cold conditions. There are likely similar mechanisms maintaining cell membranes in both stages during cold stress.

Consistency in responses to temperature stress in the sporophyte and gametophyte supports selection influencing inter-generational temperature tolerance adaptation is southern plants. Evidence of acclimation to higher temperatures in HCMS, reduced variation in HCHPL, pollen grain dormancy, and inter-generational correlations were all observed for plants from the south and could be a result of stronger selective pressures in the south.

Plants in the south experienced extremely high temperatures regularly and the maximum is much higher than temperatures in the north. The north did reach extremely low temperatures, but the plants were likely dormant during those times or covered in snow, meaning that the lower temperature limits that northern and southern plants experience might not be that different. Therefore, southern plants have greater potential for temperature-based selection to occur and thus adaptation of temperature tolerance mechanisms. The lack of coordinated response to temperature stress in the northern plants suggest that traits facilitating temperature tolerance are not important for survival. Another explanation is that horsenettle hasn’t been located in MN long enough for selection to act on the populations All populations included in this study were located toward the edge of the range for this species. Time for selective pressures to act on the populations in the north may be insufficient for local adaptation to occur. The first record of *Solanum carolinense* in Minnesota is from 1939 and in Houston County 1975 (University of Minnesota, Minnesota Biodiversity Atlas, Bell Museum - <https://bellatlas.umn.edu/collections/listtabledisplay.php>). The first record in Texas is from 1917 and the closest record of horsenettle to Collin County is from 2011 (University of Texas Austin, Billie L. Turner Plant Resources Center - <https://prc-symbiota.tacc.utexas.edu/collections/list.php>). Since horsenettle has been located in both locations for relatively similar amounts of time, the strength of temperature selection is likely driving the divergence we observed.

**Conclusion**

Our results showed evidence of local adaptation due to temperature acting as a selective pressure. At first glance, the mixed outcomes of this study do not completely support our predictions, suggesting that the response to temperature tolerance is less coordinated than we originally anticipated. However, upon recognizing that temperature might not be a strong selective pressure in the northern region, we concluded that our results support evolved responses to temperature stress in the south, but not in the north for most traits. The measurements of chlorophyll fluorescence did provide some evidence that populations from areas with larger thermal ranges, such as those in higher latitudes, have more variation and possibly more phenotypic plasticity, which is consistent with the climate variability hypothesis.

There was evidence of potential for inter-generational temperature tolerance adaptation in southern plants as well. High temperatures have likely selected for higher proportions of low-ROS pollen, which may impact sporophytic physiological processes such as photosynthesis. To fully understand the link, or lack of link, between gametophytic and sporophytic temperature tolerance, we must understand the molecular underpinnings driving these responses and how they evolve.

These results could inform restoration efforts by changing the way we think about seed sourcing and adaptive potential in a rapidly changing environment. Seeds from the south may have evolved stress responses to temperature that are lacking in northern populations. The evidence for the two-basket model in a wild species is also a novel finding that could add to our perception of the influence gametophytic traits have on a species persisting in extreme environments.

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