**INTRODUCTION**

The objectives of this study are to 1) determine if there are genetic differences in seeds sourced from a range of distinct environments across the range of *G. triflorum* 2) determine if detected genetic differences are greater in traits important to adaptation, and 3) assess what mechanisms may be contributing to genetic differences

***Study species —*** This project focuses on *Geum triflorum (*Pursh) or Prairie Smoke, which is an herbaceous perennial native to the Great Plains of the U.S and Central Canada (Fig. 2). *G. triflorum is* a member of the Rosaceae family and an allohexaploid, with a phylogeny strongly affected by reticulate evolution and genome duplication (Gajewski, 1959; Smedmark and Eriksson, 2002). *G. triflorum* consists of a basal rosette of leaves that undergo seasonal dormancy, but sometimes appears to remain evergreen. It is one of the earliest flowers blooming in spring, with flowering occurring from March until June. Flowers consist of one or more inflorescences containing three florets that are pale-pink to purple, with closed corolla.

**MATERIALS AND METHODS**

***Field collection of Geum triflorum—***In the spring of 2015, seeds from 22 populations of *G. triflorum* were sampled across much of the species’ natural range. Nineteen populations were sampled spanning three distinct eco-regions; including eleven populations representing the Great Lake alvars (GLA) region, two populations from Manitoba alvars (MBA) region, and six populations from the Prairie (PRA) region (Figure 1, Table 1). Forty open-pollinated maternal seed families were collected along a 100m transect within each population (see details in Hamilton and Eckert 2007). In addition to field collections, three bulk seed collections were provided by commercial growers (SD-PMG, MN-PMG) and the USDA (WA-BLK) from the prairie region (Figure 1, Table 1).

***Common garden experimental design—***A common garden experiment focusing on trait differentiation across regions was established on November 7, 2015 at North Dakota State University. Seeds from the 22 sampled populations were planted in 12 randomized blocks. For field collected populations, ten maternal seed families were planted per population, including 12 individuals per maternal seed family. For bulk-seed collections, 24 seeds were planted including two replicates per block for each source (Table 1). The total number of individuals seeded for the experiment was 2348 (Table 1) Seeds were treated with a .02% PPMTM fungicide treatment and planted in cone-tainers (Stuewe & Sons 158mL) filled with Sungro horticulture mix soil. Plants were re-potted into 1014mL mini-treepot containers (Stuewe & Sons) following approximately two months of seedling development. Greenhouse conditions were maintained at a 15:9 hour daylight to darkness photoperiod with supplemental light from halide lighting at a measured flux density of 03383 mmol m2 s-1 and temperatures between 18.3°C and 23.9°C throughout the course of the experiment. Plants were watered bi-weekly, and were provided a slow release fertilizer mix (Osmocote 14N-14P-14K) intermittently throughout the course of the experiment. In May 2016, seeds which had germinated and established were transferred to a permanent outdoor research facility at the Minnesota State University Moorhead Regional Science Center (Table 1, 46.86913N, -96.4522W). The randomized blocks were kept in the same arrangement. The number of individuals established ranged from 5 to 108 per population, with the exception of AB-RL, which exhibited 0% emergence in the greenhouse (Table 1), and were planted directly into the ground through cutouts in a weed barrier to limit competition.

***Morphological Measurements—*** Leaf morphological trait variation was assessed for all surviving individuals from the greenhouse prior to transfer to the permanent outdoor research facility. One leaf was randomly sampled per individual (n=1396) and photographed on a 1cm2 grid to assess morphological trait differentiation in midvein length, si nus depth and mini leaflet presence and morphology. Midvein length was measured as the total length of the primary vein per sampled leaf. Sinus depth refers to the depth of the margin between the apex lobe and the next nearest lobe. Mini-leaflets, defined as??? were assessed on a binary scale; including presence or absence of mini leaflets, followed by assessment of shape as lobed or non-lobed leaflets. All measurements were assessed using ImageJ (Schneider et al 2012).

***Physiological Measurements——*** To evaluate genetic difference for physiological traits across regions and populations, specific leaf area, chlorophyll fluorescence, leaf dry matter content, and stomatal conductance were assessed on a subset of individuals during the summer of 2018.These traits were evaluated as they encompass a physical representation of the tradeoff between efficiently acquiring resources via carbon sequestration while minimizing water loss through transpiration. Between one to five individuals were randomly chosen per population. Due to the unbalanced nature of population representation per region within the outdoor permanent common garden experiment, this led to between 9 and 56 individual measurements per trait, per region (Table 2).

Specific leaf area (SLA), which is calculated as a ratio of leaf area to dry mass, were measured over one day using a LI-3000C (*Li-Cor Biosciences*) portable area sensor for 99 individuals (~5 individuals per population for a total of 33 Prairie individuals, 56 Great Lake alvar individuals, and 10 Manitoba alvar individuals were measured). The surface area of one randomly selected mature leaf was taken alongside fresh and dry mass. Leaves were dried for 68 hours at 50°C, following which dry mass was estimated. SLA is calculated as dry mass per unit leaf area. An additional measurement of leaf dry matter content (LDMC) was concurrently calculated with this data, as LDMC is calculated as the ratio of fresh mass to dry mass.

Chlorophyll fluorescence was measured over a single 90-minute period in the field common garden using a CCM-300 (Opti-Sciences) on 98 individuals: (~5 per population for a total of 33 Prairie individuals, 56 Great Lake alvar individuals, and 9 Manitoba alvar individuals were measured). The CCM-300 records emission ratios of 700 and 735 nm (red and far red wavelengths) as a proxy for chlorophyll content (Gitelson et al. 1999). Here, we use chlorophyll fluorescence as a proxy for individual leaf chlorophyll content.

To quantify integrated water-use efficiency we used carbon isotope composition as measured by 𝛿13C (Farquhar 1989). Leaf samples from approximately five individuals per population (53 Great Lake alvar individuals,9 Manitoba alvar individuals, and 31 Prairieindividuals) were sampled from the field common garden and oven-dried at 55°C over a twenty-four hour period. Following this, leaf samples were homogenized into a fine powder using a TissueLyser II (Qiagen, Hilden, Germany) and 4-5 mg of each sample were weighed and placed into a tin capsule **(**Costech, Valencia, CA, USA**)** for 13C isotope analysis using a continuous flow isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at UC Davis Stable Isotope Facility (Davis, CA, USA). To assess the repeatability of isotope measurements across samples, 23 technical replicates were evaluated. A correlation of r=0.793 across technical replicates provides increased confidence in the repeatability and precision of the assay. Reported 𝛿13C values are expressed as relative to the Vienna Pee Dee Belemnite.

***Stomatal Measurements—***Stomatal impressions were taken from both the adaxial and abaxial leaf surfaces by using a thin layer of Newskin “liquid bandage”. Impressions from two leaves per individual were taken based on methods described in Greer et al. 2017 (N=650, 417 Great Lake alvar, 91 Manitoba alvar. And 142 Prairie, X per population). Impressions were mounted onto slides and photographed using photographed using a Zeiss Stereo Discovery (V8) digital microscope with a Canon Rebel T3 E0S 1100D digital camera.. Stomatal density was determined by adding together the number of stomata from the abaxial and adaxial side and then dividing by the chosen grid of 0.32 x 0.42mm**.** Guard cell length was measured in ImageJ (Schneider et al., 2012) and is reported as the average of three stomatal measurements from each leaf surface. Stomatal area index was also calculated as the product of guard cell length and stomatal density (Bertel et al 2016).

To evaluate regional differences in additive genetic variation, physiological traits were measured on a subset of individuals planted within the field common garden during the summer of 2018***.*** Measurements on one to five individual per population were conducted to assess chlorophyll fluorescence, specific leaf area, leaf dry matter content, and stomatal conductance. Due to the unbalanced nature of population sampling per region, this resulted in between 9 and 56 individuals evaluated per region (Table {f}).These traits were evaluated because they encompass a physical representation of the tradeoff plants face—between efficiently acquiring resources via sequestering atmospheric carbon while minimizing stresses such as water loss through transpiration.

Specific leaf area (SLA, the ratio of leaf area to dry mass) was quantified using a LiCor LI-3000C portable area sensor for 99 individuals in the field common garden (~5 per population for a total of 33 Prairie, 56 Great Lake alvar, and 10 Manitoba alvar individuals). One leaf per individual was removed to calculate length and area. The fresh mass of each leaf was recorded, and then following an oven-drier incubation of 68 hours at 50°C a dry mass was taken.

Chlorophyll fluorescence was measured over a 90-minute period in the field common garden using an Opti-Science CCM-300 on 98 individuals: (~5 per population for a total of 33 Prairie individuals, 56 Great Lake alvar individuals, and 9 Manitoba alvar individuals). The CCM-300 records emission ratios of 700 and 735nm wavelength (red and fared) light as introduced by Gitelson et al (1999). Gitelson et al established a linear relationship between the ratio of fluorescence at 735 and 700nm wavelengths and chlorophyll concentration when leaf tissue was excited with 430 nm wavelength light. Chlorophyll fluorescence as a proxy for concentration indicates how much investment plants put in to individual leaves.

Stomatal conductance, measured in mmol m-2 s-1, was collected from 99 individuals (~5 per population for a total of 33 Prairie individuals, 56 Great Lake alvar individuals, and 10 Manitoba alvar individuals) using a Decagon SC-100 Porometer between 09:00 and 11:30am over the course of five days (8/8/18-8/12/18) with one from each population per day to control for day to day variation. A subset of 10 individuals was sampled an additional 8 times to assess repeatability (repeatability was determined to be .338). Stomatal conductance records transpiration rates by measuring water exiting stomata over 30 second intervals. The rate of conductance is intrinsically linked to transpiration as well as gas exchange (CO2 acquisition), which encompasses one of the most important tradeoffs in plant growth.

***Stable Isotope Analysis—***To quantify integrated water-use efficiency we used carbon isotope composition. Leaf samples from approximately five individuals per population (53 Great lake alvar individuals,9 Manitoba alvar individuals, and 31 Prairieindividuals) were sampled from the field common garden and oven-dried at 55 ℃ over 24 hours. Following this, leaf samples were homogenized into a fine powder using a TissueLyser II (Qiagen, Hilden, Germany) and 4-5 mg of each sample were weighed and placed into a tin capsule **(**Costech, Valencia, CA, USA**)** for 13C isotope analysis using a continuous flow isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at UC Davis Stable Isotope Facility (Davis, CA, USA). The reported 𝛿13C is expressed relative to the Vienna Pee Dee Belemnite.

***Statistical Analysis—***

***Aim 1: Assessing Genetic Difference of Quantitative Traits—*** Mixed-effects models were conducted to assess genetically-driven difference in phenotypic variation observed in the common garden. Region was considered as a fixed effect and population was a random effect. Bartlett test for homogeneity of variance and Shapiro-Wilk test for normality were run to verify validity. Of the 14 traits assessed, specific leaf area (SLA) and stomatal ratio were log-transformed to meet normality assumptions In addition, a square root transformation was used for stomatal density, stomatal conductance, both adaxial and abaxial stomatal area indices (SAI), and sinus depth. Midvein length did fail both the Shapiro-Wilk test and Bartlett test, but was visually assessed as normal.

To determine if trait variation was genetically drive based on source region and population, mixed-effects models were conducted using the lmer function in the lme4 package (Bates et al., 2015; R Core Team, 2018). Omnibus ANOVAs of mixed models were conducted to assess significant differences between fixed effects (Table 2). lmerTest package [cite] was used to extract P-values. P-values inform whether there is a significant linear effect of the fixed effect, and whether the random effect helps the model fit better. Region was the only fixed effect considered, and population was assessed as a random effect, as populations sourced were a random representation total number of populations in each region. All statistical tests were run in R (R core team 2016).

***Aim 2: Examining potential factors driving differentiation of quantitative traits—*** {Climate regression chunk here} Climate data for source population was 30 year average data for 23 climate variables corresponding to source latitude, longitude, and elevation. Data was sourced from ClimateNA database (cite). Climate varied primarily on a gradient of temperature and precipitation, so variables selected to compare effect on traits included [LIST CLIMATE VARIABLES USED HERE].

[Regression analysis of climate as predictor for observed traits]

***Aim 3—Does transfer of seed across region and climate difference affect fitness? —*** {new statistical approach writing chunk

**RESULTS AND INTERPRETATION**

***Physiological traits—***Of the physiological traits, there were mixed results. Significant differences as a result of source region were shown for three of the four traits. There was a significant difference in chlorophyll fluorescence amongst Great Lake alvar- and Prairie-sourced individuals, with Prairie- and Manitoban alvar-sourced individuals investing more chlorophyll on average than those from the Great Lakes alvars (n=96, p = .0000696). There was no significant difference in Specific Leaf area amongst source regions (n=97, p = .532). There was a significant difference in leaf dry matter content (LDMC) between Great Lake alvar- and Prairie-sourced individuals, with Prairie plants having a larger dry mass on average than those from the Great Lakes alvars, and Manitoban alvar individuals in between (n=97, p = .00042).

***Stomatal traits—*** Stomatal conductance was showed no significant difference among plants (n= 96, p = .2082). There were mixed results for source region on stomatal arrangement characteristics.There was a significant difference in stomatal ratio by region: Manitoban alvar-sourced individuals had significantly more stomata on the adaxial side than both the Prairie and Great Lake alvar-sourced individuals (n=218, p = .0000645). There was significant difference in stomatal density by region, with significant differences being observed between Prairie-sourced individuals from both Great Lake alvar- and Manitoba alvar-sourced individuals (n=517, p =.0000). Average size of stomata was also different by region, with Prairie individuals having significantly larger stomata than both alvar-sourced populations (n=604, p= .0000). Prairie individuals also had a significantly greater stomatal area index compared to both Manitoba alvars and Great Lake alvars (n=507, p=.0091). **Climactic pressures on stomatal traits varied as well.**

***Isotope analysis—*** 𝛿13C was significantly different between Prairie and both Alvar regions, with Prairie having the lowest discrimination factor, and therefore the lowest water-use efficiency (n=69, p=0.0000). Manitoba-sourced individuals had the lowest 𝛿15N, significantly different from Prairie (n=69, p=0.0043). Prairie and Great Lake individuals showed no difference in 𝛿15N content. Of the stomatal traits, SAI was shown to have significant impact on 𝛿13C for Prairie and Great Lake individuals (n=30, p=0.0068 & n=31, p=0.0055).

***Morphological traits—***There were significant differences in leaf sinus depth (n =1391, p = .03441), mid vein length (n = 1391, p <0.0001), and average leaf area (n = 427, p = 0.03). Significant difference in leaf sinus depth was observed between the Great Lake and Prairie individuals. Mid vein length was significantly different between Great Lake and Prairie individuals, and Great Lake and Manitoba individuals. Average Leaf area was significantly different between Great Lake and Prairie, as well as Manitoba and Prairie.

***Climate data—***The source climates differed primarily on two axes: precipitation and temperature (fig x). The highest loading eigenvalues on the principal component analysis for axis 1 were mean annual precipitation (MAP), precipitation as snow (PAS), and longitude. The highest loading eigenvalues on the second axis were beginning frost free period (bFFP), Hargreaves reference evaporation (Eref), and mean warm month temperature (MWMT).

**Climate correlations—**Mean annual temperature was highly correlated to other temperature traits but is a more biologically relevant variable so comparisons of the temperature axis were assessed against MAT. Stomata size, and ratio were significantly correlated to MAT. MAP was correlated to stomatal size, density, ratio, and area index. Eref was highly correlated to stomatal size. All comparisons are included in Table 4. Comparisons of climate variables separated by region are summarized in Table 5.