qGen3: Increasing our sample size for study of Additive genetic variance of fitness in *Echinacea angustifolia*

Purpose

The goal of qGen3 is to duplicate the qGen2 experiment. The purpose the qGen3 and qGen2 experiments is to compare the evolutionary potential of two remnant prairie populations of *Echinacea angustifolia* by estimating the additive genetic variance of fitness under two mating scenarios (cf. Fisher's fundamental theorem of natural selection). The first scenario is a paternal half-sib experimental crossing design with all matings among plants from two "core" sites: SPP and LF (core x core). The second scenario has the same paternal half-sib crossing design with sires from the core and dams from sites peripheral to the core. We aim to quantify the extent to which small peripheral remnants enhance or diminish the capacity for evolution by natural selection. The crosses performed (core x core, core x periphery) in this experiment will quantify additive genetic variance for fitness in each site and each experimental group. Additionally, we will test for differentiation among families; do progeny from sires differ after accounting for maternal (dam) effects?

We hypothesize:

1. Additive genetic variance exists for fitness in *E. angustifolia*.
2. Additive genetic variance differs between two mating scenarios: crossing within a core site and crossing between the core site and its peripheral sites.
3. Progeny fitness differs among sires after accounting for maternal effects.

Crossing Design

We used a standard paternal half-sib crossing design where one pollen donor (sire) in nature was crossed with four randomly chosen maternal plants (dams) in a common garden experimental plot. We chose plants at Landfill (LF) and Staffanson (SPP) to act as sires. We chose 52 sires from SPP and 25 sires from LF and planned to cross each of them with two dams originating from the same core site as the sire, and two dams originating from sites peripheral to the core site (n = 108 dams). Each dam received pollen from two different sires per head. We replicated this design for two sites; Landfill (LF) and Staffanson (SPP) -- the two largest sites in our study area (n = 302 crosses). The number of dams originating from the LF that flowered in 2015 limited the numbers of crosses performed for the LF group.

We chose sires from plants that flowered at Landfill and Staffanson core sites in 2015. At each site we attempted to choose a random sample of flowering plants that covered the entire spatial distribution of plants at the site and represented different clusters of flowering plants. *Echinacea* flowering at the Landfill site occur on two main hills. At Staffanson, *Echinacea* are distributed in patches, occurring in numerous dense and sparse patches across much of the preserve. Because sites differed we used methods to choose sires specific for each site.

Selecting sires at the Landfill:

> We aimed to prioritize sires that were used in the 2013 qGen2 experiment, however there was little overlap between suitable qGen3 sires and sires from qGen2. We revisited each of the qGen2 plants using the 2013 stake data, and assessed whether they were suitable to be used as qGen3 sires. To be suitable the plants had to not only be flowering this year, but also have ~60 immature florets to ensure that we could collect several vials of pollen on 2-3 days over the course of a week. We identified plants that fit these criteria after an initial staking session, and then added plants as needed to have ~57 sires across the East and the West hills. All of the Landfill sires were also part of normal phenology data collection. In total, there were approximately 262 plants flowering at the Landfill in 2015. As it turned out, the number of Lf core maternal plants in p1 limited our crossing design and the number of sires needed. Therefore, we randomly chose 25 of the initial 57 plants to act as pollen donors (see Figure 1). Ultimately, only 2 of our 25 LF sires had been used as sires in qGen2.

Selecting sires at Staffanson:

> We chose sires representing flowering plants distributed across the entire expanse of Staffanson prairie preserve. We chose plants along a "U"-shaped transect through the preserve. We aimed to include equal numbers of sires from the North and South sides and from the East and West burn units (see Figure 2). Before walking through each of the four sections, we estimated the number of flowering plants we would encounter in that section. Then we calculated the proportion of plants in that section that would yield a total of 13 selected plants. We then walked the path and after we encountered a flowering plant, we selected the plant at random with the probability of the proportion calculated for that section. Sires from qGen2 were automatically chosen. Afterwards, we eliminated non-q2 excess plants at random to bring the number down to 13. Eight of the 52 sires from SPP were also sires used in qGen2. None of the sires overlapped with plants on the permanent Echinacea Project transect.

We chose maternal plants from *Echinacea* plants flowering in 2015 in a common garden experimental plot that originated as seed from the core and peripheral sites. The maternal plants of our chosen dams (granddams to the achenes generated by our crosses) flowered at the Landfill, Staffanson, and peripheral sites in 1995, 1996, and 1998. Our dams represent four pedigree groups (as listed in the cg database): 1. core Staffanson (SC), 2. Staffanson peripheral sites (SP)—rrx, rrxdc, nrrx, btg, 3. core Landfill (LC), and 4. Landfill peripheral sites (LP)—alf.

The smallest of these groups was flowering plants originating from Landfill core. We aimed for a balanced design across all groups, but ended up with a balanced design within site since the Lf core group was so small. (Information about dam selection, number of flowering plants in each group, and experimental design, see the R scripts used to design the experiment: crossingDesignQ3_landfill.r, crossingDesignQ3_spp.r, damSelex1.r, damSelex2.r, damSelex3.r, damSelex4.r, setUpLfDams.R, setUpSppDams.R, makeMapForChoosingSiresLf.R).

We visited each selected maternal plant on July 17, 20, and 21 to check if it was suitable for the experiment. Heads that had already begun flowering 3-4 days before this date, looked deformed, or appeared unlikely to develop with enough florets for our desired sample size (30 achenes per half of head) were deemed unsuitable for the experiment. We bagged selected heads with pollinator exclusion bags to prevent natural pollination for the duration of their flowering. We marked each maternal plant with a flag labeled with a unique damId, and used different flag colors for each of our sites; LF flags were white and SPP flags were pink. Most dams had just one head (Table 1), but dams with multiple, suitable heads received pollen from up to 10 sires (two per head). Heads that were not used in the experiment that were on the same plant as selected maternal heads were unbagged and open pollinated to simulate levels of seed set that plants might experience in nature.

Head Count	Landfill	Staffanson
1	30	47
2	7	16
3	2	3
4	0	2
5	0	1

Table 1. Number of dams from each site with their head count. Head count refers to all heads on a plant that were used in the experiment, not absolute number of heads per plant.

Pollen Collection

Once sires were chosen, we placed mesh bags onto flowering heads to exclude pollinators. We collected pollen from each sire every other day as flowering progressed. We collected pollen by sandwiching the anther between the lip of a microcentrifuge tube and a toothpick. We gathered pollen by dragging the toothpick from the base of the anther up to the top, effectively squeezing the pollen out of the anther and into the microcentrifuge tube. We generally used 3 microcentrifuge tubes per head per collection day and collected pollen from about 5 florets into each tube. If sires had more than one flowering head then we evenly divided pollen into more microfuge tubes during collection.

We labeled the caps of microfuge tubes in colored permanent marker with sire number in the field (see Figure 3). Marker color was coded with collection date. This enabled us to keep track of the age of the pollen in our pollen collection.

Our pollen inventory and use of vials was carefully controlled in 2015 compared to 2013. We stored pollen in the refrigerator. When needed for crossing, pollen was pulled based on what was needed for each dam on a crossing datasheet. We transported pollen in styrofoam trays to the field site**.** Stuart developed a workflow so that crossers knew from whom to receive pollen or to whom to give pollen vials if they were to be used for multiple crosses on a certain

day. On the busiest crossing days we transported pollen to the field in coolers. Hattie and Per acted as "runners"; they moved pollen between crossers and kept track of the pollen vials in coolers in the field (see Figure 4). For a few sires with very limited numbers of pollen vials, we returned pollen vials to our inventory after being used for crossing. In most cases, we discarded pollen vials after they were used each day for 1-3 crosses. We used “older” pollen supplies before more recently collected supplies, but cross failure was probably not due to pollen age or degradation (as it may have been in 2013 when we exposed individual tubes to cool/warm/cool cycles). Interestingly, Keke Roberts, a senior from Lake Forest College, found that qGen3 had a significantly greater proportion of successfully crossed achenes compared to qGen2 (31.46% in qGen3 compared to 24.43% in qGen2 based on "minFullCt" described in "X-raying" section).

We kept an inventory of how many microfuge tubes of pollen we had collected from each sire in a csv file and inventoried the pollen collection to make sure we did not run out of pollen from sires with low pollen or high demand.

Crossing preparation & procedure

Each maternal head received pollen from two different sires. We painted a line of bracts from the base to the top of the head with the paint color associated with each cross (see Figure 5). Lines were oriented N-S, thus dividing each head into an East and West half. We used a total of four paint colors (lavender, aqua, pink, and white). SPP maternal heads were painted with lavender and pink. Lf maternal heads were painted with lavender and aqua. Exceptions to painting a N/S line occurred when heads were tilted or deformed. In these cases we altered the paint line direction from N/S to ensure that each cross would result in roughly equal numbers of achenes.

We aimed to bag each head before flowering started, however in some circumstances we bagged heads after flowering started. If so, we painted all bracts subtending styles with white paint to indicate possible contamination by undesired pollen. We also used white paint to mark if we mistakenly put pollen on the wrong styles. We recorded a count white bracts on our datasheet to keep track of contaminated achenes throughout the crossing process. We did not use wire cages on top of the maternal heads. In 2013 we initially used wire cages, but removed them when heads changed shape and cages were difficult to remove without damaging styles.

We recorded all crossing information on paper datasheets that were reused throughout the crossing period. Typically each crosser was given an assignment that was 1-2 pages of datasheets. The SPP datasheets comprised 28 pages; the Lf datasheets 15 pages. On average, we crossed each head every third day after flowering began until flowering was complete (see Figure 6). Crossers recorded their initials and crossing date as a 3-digit code (717 = 17 July). We noted the approximate number of styles crossed on each date as well as the approximate number of styles shriveled "DTX" (due to crossing), "DTA" (due to age), "IDK" (I don't know) from previous crossing. We did not take action to ensure crosses were compatible or that sires were not related to dams. This being said, we did not observe any crosses that were obviously

incompatible during the crossing process. Since our pollen collection included many more vials per sire than in 2013, we sometimes used a pollen vial for only one cross. Notes on the crossing datasheets indicate if a pollen vial was "DIS" (disposed of) or "RET" (returned to the cooler to be used for another cross that day). Once all styles were crossed, we re-visited each head to ensure that styles were shriveled. We repainted subtending bracts as flowering heads began to senesce and bracts dried out and changed shape. Bags were removed after repainting.

Harvest

Heads were ready to be harvested according to standard *Echinacea* protocol (see http://echinaceaproject.org/wp-content/uploads/2013/05/harvestProtocolCG2013.pdf). We harvested qGen3 heads separately from all other *Echinacea* plants in the common garden experiment and kept the heads in egg cartons. The "letNo" identifier for each head was written inside each section of the egg carton. All heads from SPP had letNos beginning with the letter "W," all heads from LF were assigned letNos beginning with the letter "X." Harvest dates ranged from August 21 to September 21; most heads were harvested on September 10.

Dissection

Katherine, Ali, and Amy dissected achenes from all heads between September 23 and October 5. We labeled coin envelopes with letNo and paint color. Achenes from each head were divided into three envelopes: one for each cross, plus one "wht" envelope which contained uncrossed, contaminated, or ambiguous achenes. All envelopes of achenes were brought to CBG to be divided, scanned, and x-rayed.

Sampling achenes

We divided the achenes from each cross into three groups (henceforth samples), aiming for equal numbers of achenes per sample, and scanned these samples on a glass tray so that achenes could be counted later. We labeled these samples on the scanning tray 1, 2, and 3. After scanning we placed each sample from the cross into its own hand-folded glassine packet (1cm x 15cm) and affixed an adhesive pre-printed label to the packet with a unique identifier (packetId). The packetId is the envelope identifier appended by 1, 2, or 3 (ex. WM6408lav1, WM6408lav2, WM6408lav3). The labels also included a new unique, 4 digit, consecutive integer PO (planting order) ID number randomly chosen across the whole experiment.

X-raying and counting full achenes

Using an overhanging end of each adhesive label, we affixed 32 glassine packets on each of 29 sheets of paper cut to the size of the x-ray film (10" x 12"). Packets were ordered by the 4-digit PO ID, effectively randomizing all samples. We took a photo of each sheet prior to x-raying to ensure that we could read the labels on each packet. However, this was unnecessary since the laser printed labels were readable on the digital x-ray files. In order to expose the achenes

to the smallest dose of x-ray for the shortest duration we operated the x-ray machine in the dark and without the use of the x-ray film protective sleeve. Additionally, we covered the lasers in the x-ray chamber with opaque lab tape so that they would not expose the x-ray film.

On October 8th we x-rayed each sheet of achene packets at 12 kV for 4 seconds. After scanning the x-ray film, we flipped each DICOM image horizontally and saved it in *.jpg format. We named each file using the ID of the packet on the upper left of the sheet. (jpg files are stored here: Dropbox\qGen3_crossingExperiment2015\imagesXray, DICOM images are stored here: I:\\Departments\Research\EchinaceaQ3onI-Drive\imagesXray).

After we saved the x-ray images we made a quick list of the packets that had zero full achenes. We excluded these packets from planting. Additionally we marked packets that only had one full achene with a black line. These packets were planted differently (see below). All packets were labelled with a second label pre-printed with PO, and the row, and segment in which to plant them.

Katherine and Gretel made thorough counts of the number of full achenes per packet. We recorded "minFullCt" and "maxFullCt." MinFullCt (minimum full count) is a more conservative estimate of the number of full achenes from each crossing sample, while maxFullCt includes those achenes that appear neither unambiguously full nor empty. The counts made by Katherine and Gretel were compared and packets where counts differed were recounted by Gretel.

Site Preparation and management

In preparation for planting we burned p8 on October 11, 2015 (see Figure 7). Other site preparation included lopping re-sprouting ash trees as necessary while we set up meter tapes on rows to be planted. Stuart broadcast seed from the following species on November 4, 2015: grasses *Schizachyrium scoparium* and *Bouteloua curtipendula* as well as forbs *Phlox pilosa* (from a 2014 collection) and *Galium boreale* (from Ben Lee's collection). The grass mix was collected in 2015 from nearby prairie sites (nice island, Krusemark, p1, and Landfill). On April 14, 2016 Stuart broadcast the remaining 1/3 of the grass seed mixture.

Plot Layout & Planting

Experimental plot 8 was established in 2013 for the first cohort of the quantitative genetics experiment (q2). From the 62 unplanted rows in p8, we randomly chose a subset of 21 rows (ranging from row 100 to row 186) where the achenes from our crosses would be planted. These 21 rows included enough positions for all our samples to be planted in a random order as well as extra segments for any samples that could not be planted due to unsuitable segments (ie. something obstructing more than half the segment). We GPS'ed and flagged positions 697, 700, 725, 750, and 755 in each planting row (when applicable). We used meter tapes aligned with our GPS'ed flags to measure out individual segments (see Figure 8). We placed 8d (2 ½")

nails in the ground every meter along rows to mark these segments. Planting took place on October 14th and 15th, 2015 (see Figure 9).

71 packets were set aside for planting in "extra" positions because assigned segments were unsuitable or they were initially not assigned to a planting segment. There were 66 cases where individual segments were unsuitable for planting because of the existing transplant plot. In two cases a rock made placing nails impossible, so the segments on either side were unsuitable for planting. In one instance a packet was first designated as containing zero full achenes, but actually had 1 full achene. These 71 packets were planted at the end and filled all but three of our potential segments. Positions 752, 753, and 754 in row 168 remained unplanted.

Achenes from each packet were planted in a line between each nail marking the segment. We left a minimum of 5 cm buffer at the end of each segment where no achenes were planted to avoid ambiguity between segments. Our goal was to spread the achenes from each packet evenly between 5cm and 95cm. Packets with only 1 full achene were previously marked (see above). These packets were not planted across the 1 m segment but instead all the achenes were emptied and planted at the midway point of the segment (50 cm mark).

Total achene counts

Keke Roberts counted scans of total achenes in each crossing sample in the winter and spring of 2016. Keke rechecked receptacles and included any additional achenes and ray achenes in with the irrelevant achenes that were not included in the experiment (i.e. those placed in our "wht" envelopes, see above). These "wht" achenes were scanned and counted separately by Keke. Total number of achenes from each dam will be summed and included in both the qGen3 crossing experiment database and the Common Garden database.

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Appendix of Figures and Maps

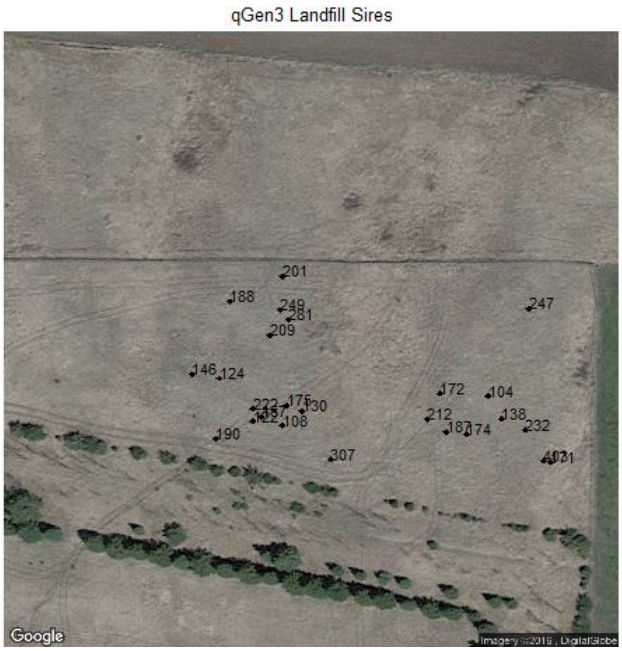

Figure 1. Locations of sires at Landfill.

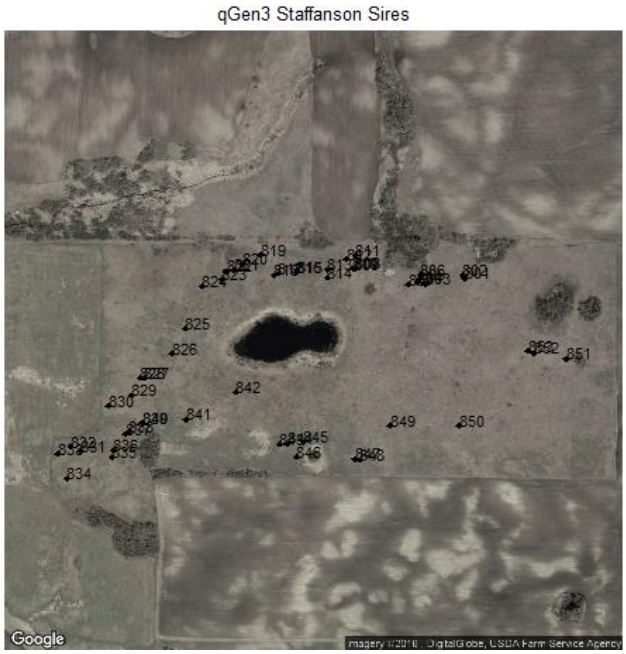

Figure 2. Locations of sires at Staffanson.

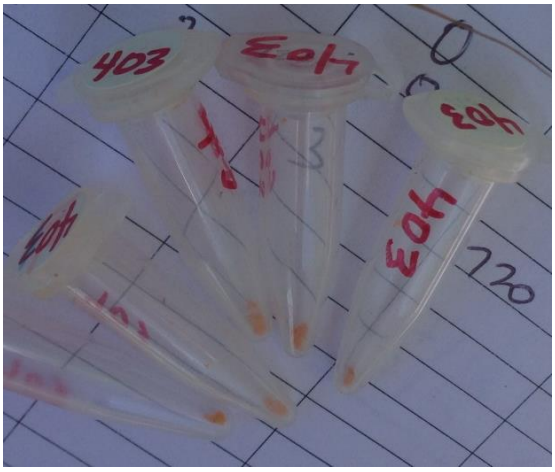

Figure 3. Pollen vials from sire 403 at Landfill.

Figure 4. Pollen delivery to crossers.

Figure 5. Painted bracts delineate crosses.

Figure 6. Amy crosses a dam in p1.

Figure 7. Burn!

Figure 8. Row setup

Figure 9. Planting packets; Ali with meter stick & tray of packets; Ali & Katherine with wagon.