qGen2: Additive genetic variance of fitness in *Echinacea angustifolia*

Purpose

The purpose of this experiment 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. 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 56 sires from each site 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 = 112 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 = 448 crosses).

We chose sires from plants that flowered at Landfill and Staffanson core sites in 2013. 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 of site differences we used the following methods to choose sires at each site.

Selecting sires at the Landfill:

We flagged all flowering plants on the East and West hills at Landfill with uniquely numbered flags. We flagged approximately 75 plants. Using a random number generator, we randomly chose a subset of the flagged plants (n=56) to serve as sires. To

ensure we could collect enough viable pollen, we removed approximately 1/8 of the plants from consideration because they were already more than halfway done flowering. In total, there were approximately 91 plants flowering at the Landfill in 2013.

Selecting sires at Staffanson:

We chose sires representing flowering plants distributed across the entire expanse of Staffanson prairie preserve. We randomly chose plants while walking along a "U"-shaped transect through Staffanson prairie preserve. We included one sire on the old road (NE corner of SPP) that is part of the existing *Echinacea* transect. All other sires were not on the existing *Echinacea* transect. We aimed to include equal numbers of sires from the North and South sides and from the East and West burn units on the preserve.

We chose maternal plants from *Echinacea* plants flowering in 2013 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, which dictated the sample size of the rest of our groups. We identified "bonus" heads for each of the other groups of maternal plants (LP, SC, SP) which we bagged in case we identified a problem with dams early on and needed to switch them out of the experiment. (Information about dam selection, number of flowering plants in each group, number of bonus heads used is in the R scripts used to design the experiment: designExpBasedOnInitalTt.R, selectSires.R, designExpFinal.R, prep crossing design workspace.R, makeResources.R, executeExperiment.R).

We visited each selected maternal plant on July 15-17 to check if it was suitable for the experiment. Heads that had already begun flowering 3-4 days before this date or 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 flagged each maternal plant, and used different flag numbers and colors for each of our crossing groups (LC = 200s, red; LP = 300s, orange; SP = 400s, lime; SC= 500s, pink). Most dams had just one head (Table 1), but dams with multiple, suitable heads received pollen from two sires 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	38	25
2	12	20
3	7	4
4	0	4
5	1	2
6	3	0

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.

Our realized crossing design was smaller than the proposed design because we had to abandon some sires (1-4 per site) and dams (9) throughout the experiment. Sires were abandoned if they started flowering too late, because they were duds, or if they did not produce enough pollen. Similarly, dams were abandoned if they were duds or in extenuating circumstances (ex: dam with ant infestation (cgPlaId 2249), possible sterile dam where no styles shriveled (most likely cgPlaId 51).

Pollen Collection

Once sires were chosen, we placed wire cages and mesh bags onto flowering heads to exclude pollinators. We initially collected pollen from each sire every other day as flowering progressed and as needed afterwards. 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 up to the top of the anther, effectively squeezing the pollen out of the anther and into the microcentrifuge tube. We generally used one 5 mL microcentrifuge tube per head per collection day. If sires had more than one flowering head or more than 20 anthers with available pollen, then we evenly divided pollen into 2 or 3 microfuge tubes during collection.

We labeled the caps of microfuge tubes in permanent marker with sire number in the field and after each collection we color-coded microfuge tubes by date. We placed stickers on the side of microfuge tubes to signify collection date (red = first collection date, orange = second collection date, etc.). Additionally, a certain paint color was assigned to each sire and that color paint was used to delineate the cross on the dam. We therefore labeled microfuge tubes with a dot of paint corresponding to each sire's cross color. This acted as a way to double check while in the field, ensuring we were crossing with the correct sire.

We stored pollen in the refrigerator. When needed for crossing, pollen was transported in styrofoam trays to the field site and remained outside for 1-4 hours. On days where we had to cross many heads we transported all the pollen to the field site in a cooler. Attempts were

made early on to use and exhaust “older” pollen supplies before more recently collected supplies.

We kept an inventory of how many microfuge tubes of pollen we had collected from each sire in a csv file. To keep the inventory updated, we recorded every tube that we exhausted on the same csv file.

We ran out of pollen from some sires. Sires with only one flowering head producing little pollen or sires that were bagged late often did not supply enough pollen for all maternal plants. When a shortage of pollen did occur, we took steps to ensure that all four maternal plants received at least some pollen from their designated sire. Once pollen was exhausted, we painted the subtending bracts of uncrossed styles with a different color of paint and pollinated with another sire to avoid differences in resource allocation between crosses.

Crossing 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 sire (Figure 1). Lines were oriented N-S, thus dividing each head into an East and West half. We used a total of six paint colors (lavender, aqua, silver, white, green, and pink). 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.

To avoid contamination from other pollen sources we bagged each head before it started flowering. We also initially fit many maternal plants with wire cages identical to pollinator exclusion in the sires (see above) but we removed many cages throughout the crossing process as heads changed size.

On average we crossed each head every fourth day after flowering began and roughly every four days until flowering was complete. Initially we gave a 5-10 heads “test crosses”-- we applied pollen to ~5 styles to determine if it was compatible -- but as more dams began flowering we were unable to continue this step because of time constraints and instead just crossed all receptive styles every time we visited a head.

We recorded all crossing information while in the field on a visor form. This information included the date and initials of the person responsible for each cross as well as any relevant notes. We chose which heads to cross each day based on their flowering phenology. Initially we used a script in R to pull up flowering heads, but there was a glitch and we discovered that we were initially skipping some dams. As a solution we began recording a return date on the visor form for each head after we visited it based on when it should be crossed next. Every day we pulled up those heads with corresponding return dates.

Once all styles were crossed, we re-visited each head to ensure that everything was shriveled. We then changed the return date for that cross on the visor form to "999" to signify we did not have to revisit the head.

We repainted subtending bracts as flowering heads began to senesce and bracts dried out and changed shape. We started repainting around the 20th of August. Repainting began systematically by visiting those plants that flowered earliest in the season and keeping track via a visor form. After a few weeks, however, repainting became a part of the harvest procedure and we debagged and repainted heads (as needed) when they were ready to be harvested.

We removed bags from heads that were repainted early on during the systematic repainting process, but due to substantial herbivory by grasshoppers that preferentially chewed off painted bracts, we waited to remove bags until heads were harvested.

Troubleshooting

We tried to fit every maternal plant head with pollinator exclusion bags before they began flowering but some heads were bagged late. In these cases, if styles were already shriveled upon initial crossing, indicating the head had already received compatible pollen, we marked the subtending bracts of these florets with a unique paint color so that these achenes would be excluded from the experiment. We recorded a count of already shriveled styles on our visor form to keep track of contaminated achenes throughout the crossing process.

Contamination during crossing could occur when the pollen applicator (a toothpick) was doubled dipped in the pollen tube. "Double dipping" contaminated the pollen source by mixing sire pollen with dam pollen, rendering the pollen source tainted for use with other dams. We noted contamination of sire pollen by writing the ID of the dam on the pollen storage tubes. We henceforth only used contaminated pollen on those dams from which it was contaminated.

Lastly, incompatibility between sires and dams did occur. We did not take action to ensure crosses were compatible or that sires were not related to dams. We noted incompatibility during the crossing process on our visor form (i.e. if styles were not shriveling) but no cross was ever officially deemed incompatible and sire assignments were never changed.

Pollen was taken from the fridge to the field many times which may have shortened the duration of pollen viability.

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 qGen2 heads on separate days than all other *Echinacea* plants in the common garden experiment. Harvest dates were August 30, September 4, September 11, and September 17. If a

qGen2 head was ready to be harvested on a non-qGen2 designated harvest date, it was immediately placed in a qGen2 designated bag and given a gBag code of "orginalGbag/newGbag" (ex: a head harvested in gBag F but stored in gBag QA received the gBag code FQA).

Dissection

We dissected each head between September 18 and October 4 to remove achenes, and put the achenes from each cross into coin envelopes. We labeled coin envelopes with a three digit ENV ID code unique and randomly assigned to each cross (ENV IDs ranged in values from 554-989). We placed all other irrelevant achenes from the head (aborted achenes, ray achenes, or ambiguous achenes that could not reliably be placed in either cross) in a third envelope labeled "extra". In those cases where there wasn't enough pollen to complete a cross and a different sire was used to pollinate the rest of the head, a fourth envelope was assigned an ENV ID and the achenes from that cross were treated separately.

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 tray so that achenes could be counted at a later date. 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 labeled the packet with a unique identifier (packetId). The packetId is the envelope identifier appended by 1, 2, or 3 (ex. 743.1, 743.2, 743.3) as well a new unique 4 digit PO (planting order) ID number.

X-ray preparation and protocol

Using an overhanging end of each label, we affixed 30 glassine packets on each of 40 sheets of paper cut to the size of the x-ray film. 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. (Based on a trial of many different inks we found that only gold sharpie showed up on the x-rays). 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.

We x-rayed each sheet of achene packets at 12 kV for 4 seconds. After scanning the x-ray film, we flipped each image horizontally and saved it in *.png format. We named each file using the ID of the packet on the upper left of the sheet. (We need to investigate if DCIM files are backed up.)

After we saved the x-ray images we made a quick count of the number of full achenes. We excluded packets from planting if they had zero full achenes.

Additionally we marked packets that only had one full achene with a black line. These packets were planted differently (see below).

Site Preparation

We chose an old-field within 400 meters of our existing experimental plot as the planting site. We cleared the site of all trees via chainsaw, weed whip, and loppers, and treated all stumps 1-2 times with Round-Up. We burned the site on October 9, 2013.

We broadcasted seed of the grasses *Schizachyrium scoparium* and *Bouteloua curtipendula* on October 24, 2013. We collected the grass seeds from 7 nearby prairie sites (pennisula, Krusemarks, CR15, CG1, LC, Back hill, Tower) and then mixed them together before broadcasting. We also stored half (2-3 grocery bags) of the seed in a plastic tub in the Hjelm house over the winter to be broadcasted in the spring.

Plot Layout & Planting

After burning, we flagged a grid across the field using a GPS survey station with previously determined points. The grid was laid out in 5 x 5m blocks. Blocks were deemed unsuitable for planting if they were <15m from a treeline to the south or the areas of lower elevation (approximately 2 m lower than the highest sections of the plot). We placed flags directly at GPS point by pushing the pole into the ground and placing the flag at the center of the indent. After flagging all the suitable planting blocks, we had a total of 85 planting rows, each spaced 1 m apart. Planting rows varied in length; the longest rows spanned 58 m, from positions 697 to 755.

From these 85 rows, we randomly chose a subset of 23 row locations where the achenes from our crosses would be planted. These 23 rows included enough positions for all our samples to be planted in a random order as well as 2 extra rows 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. Planting took place on October 24th and 25th 2013. We placed 16d (3 ½") nails (confirm this at Farm) in the ground every meter along rows to mark these segments. There were six cases where individual segments were unsuitable for planting because a large rock was obstructing the segment or there was already a transplant plot; we set-aside the 31 packets designated for these positions and planted them in a different row.

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 where there was 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.

We planted the first 6 rows on October 24th (104, 109, 110, 111, 121, 123). Despite windy conditions, we planted the remaining rows on October 25th.

Laboratory Work

Xray and scan images of crossing samples were counted by volunteers throughout the fall and winter of 2013. Two pairs of volunteers independently counted full achenes from X-ray images and these counts were then compared for accuracy. Although in most cases achenes were clearly full or empty, there were 83 samples with ambiguous achenes which could not be classified as full or empty. These cases were reconciled by creating two columns in our database: MinFullCount and MaxFullCount. MinFullCount is a more conservative estimate of the number of full achenes from each crossing sample, while MaxFullCount includes those achenes that appear neither unambiguously full nor empty.

Volunteers counted scans of total achenes in each crossing sample once and a random subset of these images was recounted to ensure the counts were accurate. If the scan was missing or incomplete, achenes were counted from xray images. (Ask Lydia how many scans were missing or incomplete.)

Irrelevant achenes that were not included in the experiment (i.e. those placed in our extra envelopes, see above) were scanned and counted separately from those included in the experiment. Total number of achenes from each dam will be summed and included in both the qGen2 crossing experiment database and the Common Garden database.

Recommendations for future crossing experiments

The two largest issues in executing the crossing experiment were shortages of pollen and incompatibility. Shortages of pollen occurred not only because we exhausted all available pollen from a given sire but also because we exhausted all viable pollen from many sires. Although we have estimates of the duration pollen can stay viable in the refrigerator (approximately 9-11 days), towards the ending of the crossing experiment we were using pollen that was more than two weeks old. For future crossing experiments, a batch of pollen collected early on should be frozen to ensure that viable crossing can take place between asynchronously flowering sires and dams. Furthermore, although we kept an inventory of pollen, we could have been more stringent in keeping tabs on which sires we had a lot of pollen for and which sires were running low. This would help ensure that all dams could receive equal amounts of pollen from their designated sire.

Incompatibility between sires and dams did occur (as evidenced by the x-ray images) and was noted often in the field during crosses. In the future, we should have a plan for how to deal with incompatibility. If crosses seem incompatible after their first attempts we should change sires. One way to help with the issue of incompatibility would be for people to be assigned to certain dams throughout the crossing process. By following dams over the course of the season one would be more certain they are witnessing an incompatible cross. Conversely, when multiple people visit a head they are often likely to say the cross seems incompatible but apply more pollen just in case the previous person somehow didn't execute the cross adequately.

Pollinator exclusions bags worked effectively on sires and dams. From anecdotal observations, wire cages seemed to improve pollinator exclusions, however the cost was possible damage to styles. When only collecting pollen from flowering heads (as is the case with sires) cages should continue to be used, however a less damaging option should be fashioned for dams in the future. Additionally, pollinator exclusion bags should not be removed from heads until the heads are harvested to avoid herbivory by grasshoppers. Grasshoppers ate many painted bracts, rendering some achenes ambiguous or unusable in our crossing experiment.

Silver paint was difficult to see on the bracts, especially as the season progressed and bracts changed shaped. Silver and lavender were especially difficult to distinguish when on the same head. Avoid using silver in the future.

Lastly, although we did not have much flexibility in planting days for this experiment, in the future planting should not take place on windy days. The high winds during our planting made it extremely difficult to pour/sprinkle achenes on the ground and slowed down the entire planting process. Also, the flimsiness of the glassine sleeves in the wind slowed planting. A heavier weight, more rigid material may have performed better.

Acknowledgements

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Appendix of Figures and Maps

Figure 1. Example of painting on each dam to delineate crosses. Lavender and aqua painted bracts subtend and surround florets crossed with an assigned sire. White bracts signify styles that were contaminated with other pollen (Photo courtesy of Lydia English *paintedHeadExample.jpg*).

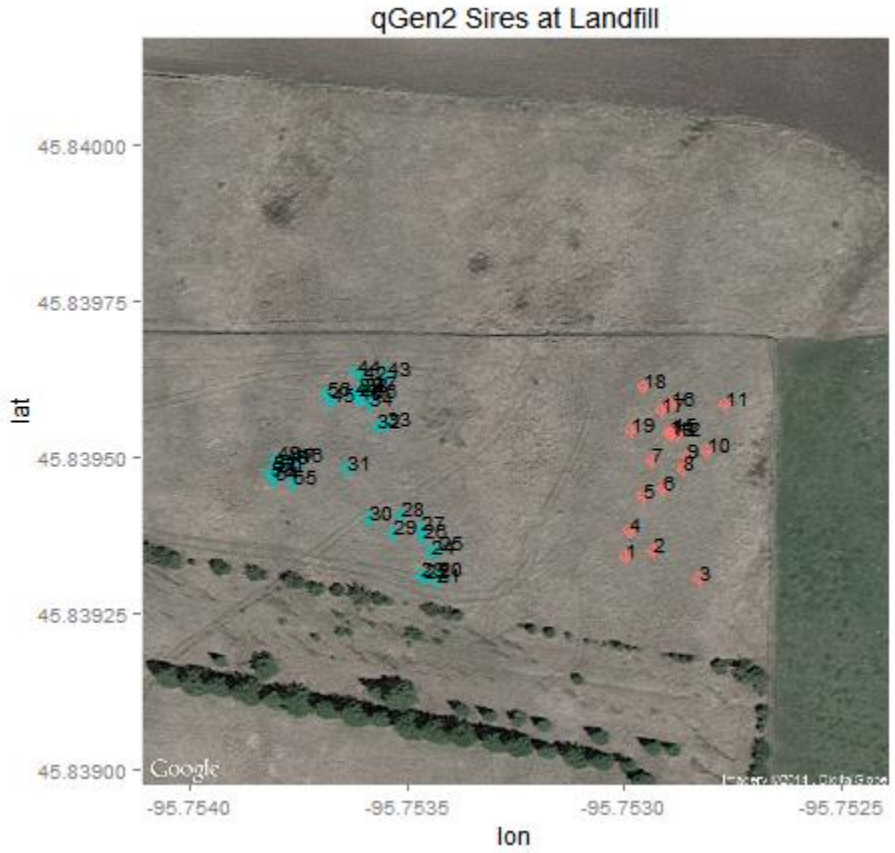

Figure 2. Locations of sires on East and West hills of Landfill.

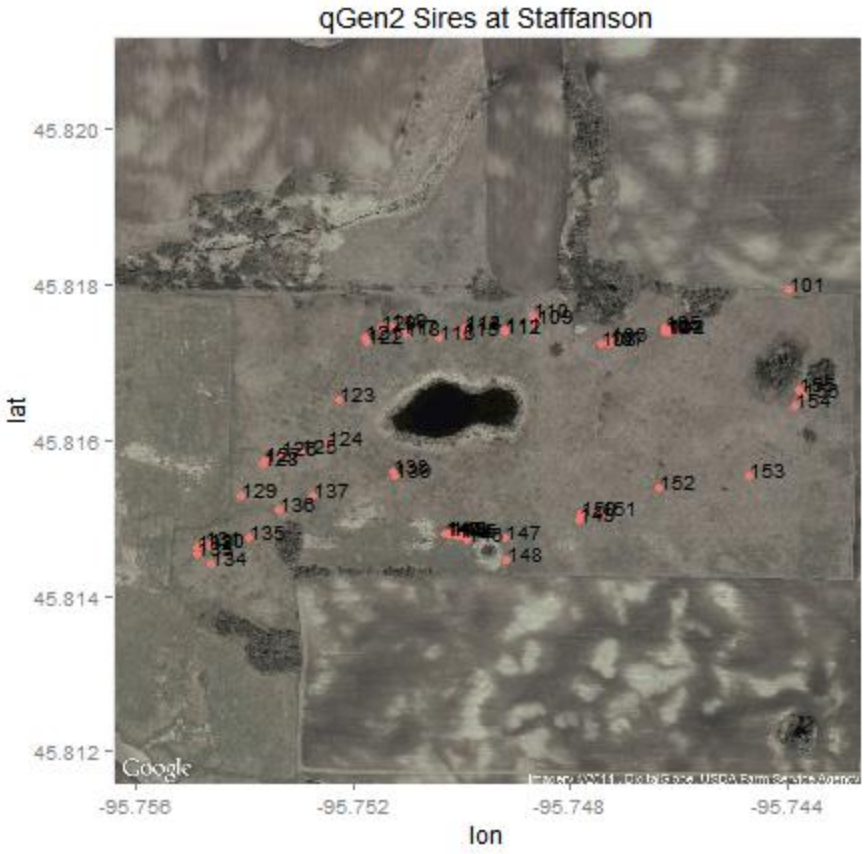

Figure 3. Locations of sires at Staffanson Prairie Preserve.

We would like to include Staffanson & Landfill maps of Grand-Dams, however survey data from early years has yet to be georectified.

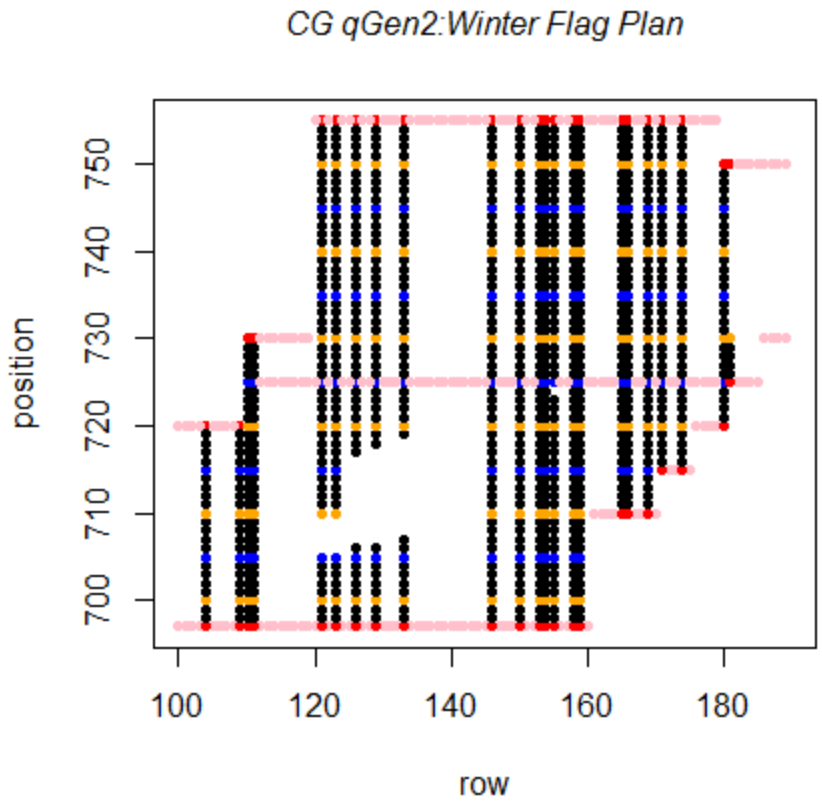

Figure 4. Map showing 2013 planting locations in cg8: qGen2 experimental plot in the South Field. Black dots are locations of nails that mark start and end of planting segments. Colored dots are flags.

Database Relationships

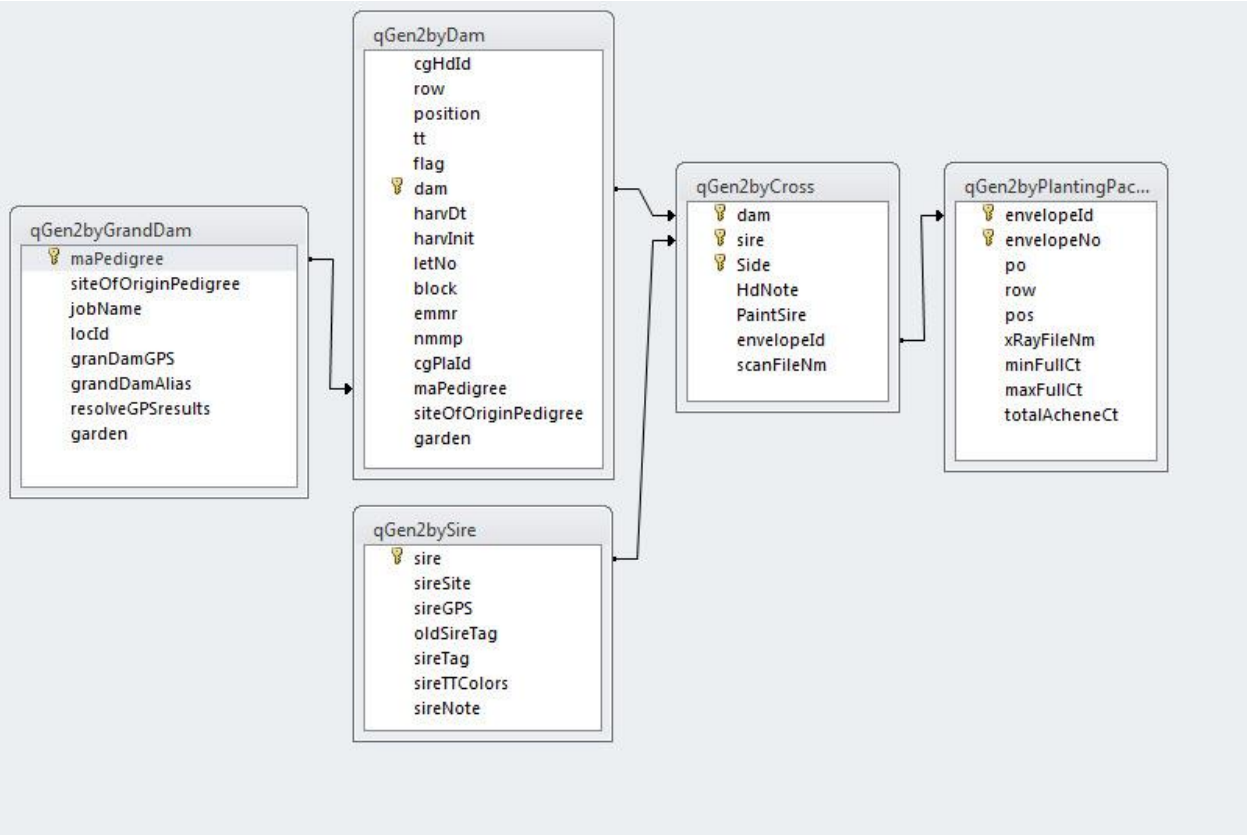

Figure 5. Relationships of tables in the access database stored here: Dropbox\qGen2 crossing experiment 2013\2013.qGen2.database.accdb