Protcols for the Tribe Collinseae Mating System Evolution Plants

Data needed from plants:

**Outcrossing rates determined byusing microsat markers to genotype progeny arrays:**  Ideally we would like to genotype 25 families per population and 20 individuals per family.  Plant material should be collected from all plants that germinate and stored in the -80 freezer for DNA extraction.

**Flower morphology Data:** The Armbruster Lab will use image analysis software to take measurements.  We will collect flowers at Stages 0-4 of floral development to send to Armbruster Lab.  For each population, we will collect at least 12 flowers per stage, taking flowers from a variety of families.

**April’s Data**:  April is performing a hybridization experiment across Collinsia species.  These trays will be clearly labeled.  Do not touch these plants without permission from April. These plants are not being used for outcrossing rates or flower collections. Also, there are some populations that are being used for outcrossing rates and flower collection, and also by April.  Data on these populations are stored in a separate binder, but should be transplanted and collected from as normal.

**Planting Seeds:** Plant 25 families per population, or as many families as we have if less than 25.  Plant 20 seeds per family or as many seeds as we have per family if there are less than 20.  For some species, such as C. parviflora, we don’t have many seeds per family – just plant as many as we have.

Bleaching: As of August 2006, we are not bleaching seeds.  Unless you are otherwise instructed, skip to the planting section.

Seed bleaching solution (from McCourt and Keith’s Methods in Molecular Biology Vol 82, Ch. 2) is a 20% bleach, .05% Tween-20 solution.  For a 100mL solution add:

20 mL bleach

80 mL diH20

50 uL (Use micropipettor) of Tween–20

Put all seeds from one family into 15mL falcoln tube.  Add a few mL of bleach solution – add only enough to completely cover seeds?Tap tube to sink seeds to the bottom of tube.

Soak seeds in solution for 5-10 minutes.  Use vacuum filtration with filter paper to remove bleach solution and rinse well with diwater.  Make sure all seeds are out of falcoln tube.  You can rinse tube with diwater to get any out that are stuck to sides. Transfer seeds onto filter paper for planting.

Planting:

Bleach Table using 10% bleach before beginning a round of planting.  Moisten Autoclaved (see autoclaving protocol) Sushine Germination mix with water in a clean bucket**.** (Bleach out bucket if soil has been sitting in it uncovered or covered for more than a week.)  Fill a 20 line tray inside a white tray with germination mix.  Pack soil down into wells, but don’t overpack.  Don’t fill lines all the way to the top. There should be 1/8” to ¼” space at the top so that seeds won’t move between wells when watered.  Cover bucket with soil in it when finished making trays.

Plant only 1 species or species with compatible germination conditions in a tray.  Keep species together as much as possible.   Plant an entire family (up to 20 seeds) in each line of the tray.  Please try to keep the families planted in numberical order.  This makes data organization much easier.

Use forceps to bury seeds about ¼” into the soil.  If you drop a seeds into the wrong line, make a note of it on the planting design datasheet, even if you don’t know where the seed fell.  Label each line with a ½ tag with species code, population code, and family number.  Tag should look like this:

                            Lin

                            BFR

                            6

Use a black Sharpie marker to do all tag labeling.  Other markers wear off.  Also fill out each line of a tray template sheet (located in binder.  If needed, print more from office dell C:/datasheets and protocols/C. species planting design template.xls)  When finished with a tray, label the tray with “C. species,” Tray #, and top/bottom.  Also put a preventative treatment label on each tray.  These labels track what type of preventative treatments have been applied by Ellen and Jessie.  Water trays lightly when finished.

Species with cold germination conditions should be placed in a Percival at 12 degree day and 7 degree night – 12 hour day.  Species with warm germination conditions should be placed in Rm 141 or a conviron with a 19 degree day and 12 night – 14 hour day.

Cold germination species:

C. callosa

C. childii

C. greenei

C. heterophylla

C. multicolor

C. parviflora

C. sparsiflora – all varieties

C. tinctoria

T. tenella

C. rattanii

C. antonina

C. parryi

Warm germination species:

C. bartsiifolia

C. concolor

C. corymbosa

C. grandiflora

C. linearis

Germination conditions unknown:

C. torreyi

When seedlings are starting to grow true leaves, they are ready to transplant.

**Transplanting**:

As of September 2006, transplant only 1 plant per family for either April’s populations or our populations.

Moisten some autoclaved Fafard No. 4 Soil with water in a clean bucket (again the bucket should be  bleached if soil has been left in it uncovered *or* for > 1 week).  Fill a tray, or however many you will need of 2.5” pots with the soil, filling each pot to the line on the pot, and lightly packing in the soil.  If you fill too much, watering will be difficult and if you fill too little, the plant won’t have enough soil to grow to full size.

With a metal spatula, make a small hole in the soil for the plant.  Gently remove the plant from the 20 line tray/96-well tray with the metal spatula and transplant it to the pot.  Try not to move soil from the 20-line tray/96well tray into the pot because you may transfer seeds with the soil.  Also try not to spill soil between wells of the 20-line tray/96-well tray for the same reason.   Bury the plant all the way to the cotyledons and pack soil around it.

Make a new full-size tag for the plant with the Species code, population code, family number and individual letter (starting with a),  plot number for April’s populations, and your initials.  The plant will get an individual letter at this point.  If it is the first plant to be transplanted and no tissue has been collected, it will be individual “a.”  The next plant to be transplanted or collected will be labeled individual “b.” Tag should look like this:

              Lin                                                                                    Py

              BFR              for Aprils                                                        SR-138

              P-8              pops                                          or                            6a

              6a                                                                                    JD

JD

Use a black sharpie marker to make the labels. **Record all individuals transplanted in the Transplanting/Tissue collection binder along with your initials**.

Water all plants when finished, even if the soil is already wet.  This packs the soil in around the roots so that they are not exposed to air pockets.  Label the tray with the date transplanted place a treatment label on the corner of the tray and move it to the conviron in Rm A136 or A137.  It will be moved into the greenhouse in 1-2 weeks.

Disinfect hands and tools between trays and whenever you touch a plant that could be diseased.  Always disinfect tools with Greenshield when you’re finished.

**Tissue collection**:

Except for the plants that were transplanted, we collect the entire seedling directly from the 20-line tray and freeze it for later DNA extraction.  Collect when seedlings have at least 2 pairs of true leaves, or when there is about a quarter’s worth of leaf area.  It’s better to err on the side of too much leaf tissue than too little. If and when we are dealing with a diseased tray, we may collect tissue earlier, when the plant has only 1 pair of true leaves.  Ask Jessie if you’re not sure what the current protocol is. If collecting tissue from transplanted plants in the greenhouse, plants should be large enough that taking a few leaves won’t hurt the plants ability to photosynthesize.  This will be at least 2 weeks after the plant was transplanted.

Before starting tissue collection for any family, check that 1 plant has been transplanted.  Also check the dead plants column to make sure the one that has been transplanted did not die.  If none has been transplanted, or the transplant died, transplant another.  Don’t reuse the same letter though – just give the next transplant the next consecutive letter.

Label a 1.5mL microfuge tube on the top and side with species code, population code, family and individual letter, using a black, VWR lab marker.  These markers are alcohol resistant and the writing will not bleed if alcohol is spilled on them during DNA extraction.   Look in the binder for the letter last transplanted or collected, and start lettering individuals with the next consecutive letter.  Wrap the sides of the tubes in tape so that we’re sure the lettering won’t wear off.

The plants will need to be kept cold once collected, so fill a cooler with icepacks or ice. (not dry ice!)  If collecting from a seedling, cut the plant just below the cotyledons or the first pair of true leaves depending on how large the plants are. A little stem tissue in addition to the leaves won’t hurt.  However, DNA is extracted more easily from leaf tissue, so don’t collect more stem tissue than you have to.  If collecting from a transplanted plant, cut a leaf tip off (for large-leaved species), or pull a few small leaf off the plant with forceps. Put the tissue into the microfuge tube and seal completely.  Put the tube on ice immediately.  Cut the stem and remaining leaves down to the soil level (for 20-line tray tissue collection) and throw away the tissue.  It’s important not to leave the stems because the dying tissue can support fungal disease.

If the tissue you are collecting is diseased/brown, or you are collecting less tissue than normal due to impending disease, mark the tubes with a red dot.  We will know later that tubes with red dots may be less than optimal for DNA extraction.

**Rinse scissors and forceps in 20% bleach water then rinse with water to make sure not to contaminate the next sample and dry BETWEEN EACH PLANT to prevent any DNA contamination from plant to plant.**  It takes very little DNA contamination to get false PCR results.  Also, bleach will break down DNA quickly so don’t forget to rinse your tools in water after bleaching them.  Every 45 minutes to 1 hour, take tissue down to -80 freezer in 163 Crawford.

Record all tissue collection in the binder.  Record in the column “whole plants collected” for tissue collected from the 20-line trays/96-well trays.  This lets us know we no longer have that plant.  For tissue collected from transplanted individuals, record in the column “letters of leaf samples collected.” Initial all tissue collection.  Also**, update the “number in freezer for genotyping” for each family.**

Tubes should be stored in a tube box labeled with the species and population.  Don’t make a new box unless you’re sure that there isn’t already a box or all boxes for that population are full.  If you need to make a new box, the box should be labeled with tape on the top and side under the latch.  Make sure to do this before putting the box in the freezer – the tape won’t stick after the box is frozen.

Put 20-line/96-well trays back in the chamber they were collected from.   Greenshield where you were working and record what you worked on in the record book before you leave.

**Plant care:**

Ellen waters and fertilizes all plants in the greenhouse.  We are responsible for watering and fertilizing plants in Percival and conviron growth chambers. Use regular tap water for all watering.

Check all plants and water every Monday, Wednesday, and Friday.  Initial and date the record sheet for each chamber when you water or just check trays.  Make sure to water well on Fridays since no one will water them over the weekend.  Also check for any diseases.  If anything looks out of the ordinary to you, contact me (Jessie) or Dr. Kalisz (office:4-4281) and Ellen York (office:8-5393).  It is very important that you inspect the plants closely and contact someone immediately if you suspect a disease, as diseases can kill an entire chamber of plants in a few days. We fertilize 20-line trays every 3 weeks after germination.  We use Scott’s 20-20-20 fertilizer at 100ppm.  To make a 100ppm solution in the carboy, add 9.6 grams of fertilizer and fill to the line with water.  Squirt each line with germinated seedlings lightly.  Don’t overfertilize!   If the line is dry, water first then lightly fertilize.

Always disinfect hands between moving in between chambers and after touching a plant that could be diseased.  Use the greenshield spray bottle in Rm A136.  To make more greenshield disinfectant, fill the spray bottle to the 2 or 3 oz mark with Greenshield Disinfectant Concentrate, then fill to the line with water.

A note about the percivals:  The Percival chambers belong to Dr. Kalisz, and it is our responsibility to keep them in working order.  Because it is relatively easy for the temperature settings dial to be moved, we record the temperature settings every time we water.  Check what temperature the dials are set at for day and night and record this on the record sheet.  Also check that the actual temp is near what it should be (+/- 2 degrees is Ok)  before you start watering.  After the door has been open during watering the temp will be high.   On Wednesdays, check that all the lights are working and empty the water collection trays under the percivals.  We also rotate trays in the percivals because conditions vary at different distances from the fan and coolers.  Every Monday, move each tray one shelf down and move the trays on the bottom to the top shelf.  Note on the watering sheet when you rotate trays.

Other chambers are owned either by Dr. Tonsor or the University.  Let me or Dr. Kalisz know immediately if there is a problem with any of these chambers.  Ellen can also usually help with any problems.

Again, it’s important to check well for diseases and alert me or Dr. Kalisz and Ellen if anything looks out of the ordinary.

It is the person who waters’ responsibility to keep Rm A136 neat and keep good records.  Percival and other chamber watering sheets are kept in the “Chamber records” binder in the lab.  When a record is filled, put it in the back of this binder and post a new record sheet.  Also, if you use the last of the paper towels, get more from the stockroom.  Feel free to purchase a few rolls so we don’t have to run to the stockroom every other week.  See below for paper towels catalog number.  Also, refill the greenshield disinfectant spray bottle when necessary.  Fill with concentrated distinfectant to 3 oz. then to the fill line with water.

**Flower collection**:

For each population, collect 12 flowers per stage for stages 0 to 4.  (Stage 0 = 0 anthers dehisced, stage 1 = 1 anther dehisced, etc) Check for the stage using your thumbs to pull the keel open, or you can use a toothpick to open the keels of small flowered species.  The flowers are stored in vials filled with 70% ethanol.  The 12 flowers can all go in the same vial. Label the vial with Species code, population, and stage.  Use a VWR Lab marker – these are alcohol resistant.   We want to collect the flowers from a variety of families, so mark on the datasheet how many flowers you collect from each family.  Don’t worry about which stages you collected from which family, but don’t collect more than 10 flowers from 1 family over all 5 stages.   Fill in hash marks on the datasheet to track how many you have collected for each stage, as well as hash marks on green label to ensure that we don’t collect more than 10 from each family.

The only exceptions for this protocol are C. parviflora, some populations of C. rattanii, and C. sparsiflora v. collina, and T. tenella.  C. parviflora and T. tenella are too small to determine the stage without a dissecting microscope, so just put a variety of stages in one vial.  Make sure to collect plenty of flowers that are still in bud as the anthers may dehisce very early in these small flowers.  For C. sparsiflora v. collina, stages 1-4 can be collected as normal, but stage 0s are too small to open the keels, so collect a variety of buds that we assume are mostly stage 0s.  The stages of these flowers will be determined during image analysis using a dissecting scope.

**Supplies**

**Greenshield:** Greenshield is kept in the lab under the sink or on the counter near the sink in a spray bottle.  If you need to make more, there is concentrated greenshield under the sink labeled either greenshield concentrate or disinfectant concentrate.  This must be diluted 1:16 with water.  For the lab spray bottle, fill to the 3 oz mark with concentrate, then fill to the fill line with water.

**Ethanol:**  70% ethanol is stored in a carboy in the lab.   If we are running low, there is 95% ethanol under the sink in the extraction room (the other 205 Clapp).  To make 2 L of 70% ethanol, add 1500 mL of  95% ethanol to 500mL of water.

**Bleach**:  Bleach is stored below the sink in the lab.  I usually estimate when making up 10 or 20% bleach solution.  A little more or less bleach won’t hurt.  However, if you prefer, there are graduated cylinders in the molecular lab so that you can measure out the exact amount that you need.

 **Item                                                                                    Supplier                            Catalog number**

Microfuge tubes                                                        Stockroom                            PL-205

Scintillation vials                                                        Stockroom                            PL-281

  (for flower storage)

Ethanol                                                                      Stockroom                            SO-250

Paper towels                                                                      Stockroom                            SU-365

Filter Paper 55m                                                        Fisher                                          09-805B

Plant tags                                                                      Ellen

Pots and Trays                                                                      Ellen

Soil (kept in 4th floor greenhouse)                            Ellen

Fertilizer (kept in GH stockroom)                            Ellen

Ellen orders all planting supplies.  Please let Jessie know as soon as possible if we are running low on something, so that I can fill out a requisition for the order.

Supplies for this project should be paid for on grant “Unraveling Dynamics of Mating Systems Evolution.”

**Species Codes:**

Ant = C. antonina

Bar = C. bartsifolia

              v. dav = variety davidsonii

Cal = C. callosa

Cc = C. concolor

Chi = C. childii

Cor = C. corymbosa

Gf = C. grandiflora

Gre = C. greenei

Het = C. heterophylla

Lin = C. linearis

Mc = C. multicolor

Pv = C. parviflora

Py = C. parryi

Rat = C. rattanii

Sp = C. sparsiflora

   v. ar = variety arvensis

   v. c = variety collina

   v. sp = variety sparsiflora

Tin = C .tinctoria

Tla = T. tenella

Tfl = T. floribunda

Tor = C. torreyi

   v. tor = variety torreyi

   v. wri = variety wrightii

Ver = C. verna

Vio = C. Violacea

Updated 9-12-07