#### Title:

A Structured Inquiry Approach to Cotyledon Phenotyping

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#### **Abstract:**

Transmission genetics labs have long been valuable hands-on explorations in undergraduate Introductory Biology and Genetics courses. While such labs are strongly analytical, they often present the scientific process as artificially linear, with a single, straightforward "right answer." To more accurately represent the scientific thought process, I have developed a structured inquiry approach to transmission genetics labs grounded in cotyledon color. Student lab groups develop their own hypotheses to answer the question "How do plants inherit cotyledon color?" based on limited, contextualized information. Each group receives its own blinded set of F2 seeds that differ from the seeds some or all other groups receive. Group members work together to define the fuzzy boundaries between colors based on actual observations, then use chi-squared tests to evaluate their initial hypothesis. Optional and advanced activities can further enrich scientific thinking in this lab by integrating hypothesis refinement, scientific uncertainty, plausible alternative explanations, and designing new experiments.

### **Learning objectives:**

Students will learn more deeply about the following concepts:

- Phenotypes and genotypes
- Monohybrid crosses
- Mendelian dominance and recessiveness
- Chi-squared testing
- Optional: Incomplete dominance
- Optional: Plant pigments
- Optional: Biosynthetic pathways
- Advanced: Dihybrid crosses
- Advanced: Epistasis

Students will practice the following scientific process skills:

- Generating a hypothesis
- Making observations
- Making judgments to categorize phenotypes
- Collecting and summarizing quantitative data

- Statistical (chi-squared) analysis
- Distinguishing between statistical and biological hypotheses
- Assessing a hypothesis based on experimental evidence (i.e., drawing conclusions)
- Optional: Inferring genotypes based on phenotypes
- Advanced: Inferring F1 and P generation genotypes based on F2 phenotypes
- Advanced: Recording unexpected observations
- Advanced: Recognizing scientific uncertainty
- Advanced: Developing new questions and/or hypotheses based on experimental results

#### Timeframe:

<u>Instructor preparation time:</u> About two or three hours to gather and prepare materials, assuming that nothing (including seed sources) needs to be ordered from suppliers. Preparing labeled, blinded seed packets is the most time-intensive task.

<u>Instruction and student work:</u> About four hours of class time, distributed unevenly across two or three weeks, depending on the species of seed used. Optional or advanced activities will require additional time. Observations will be brief (less than twenty minutes) until the majority of seeds have germinated, so other lab activities can be carried out during this time.

#### List of materials:

The classroom will need:

- F2 generation seeds from controlled crosses with distinct leaf color traits, for example:
  - o Carolina Biological's Tobacco Seed, Green: Albino 3:1 (#178400)
  - Carolina Biological's Tobacco Seed, Green: Yellow Green: Yellow 1:2:1 (#178410)
  - Carolina Biological's Tomato Seed, Green: Yellow Green: Yellow 1:2:1 (#178670)
  - Carolina Biological's Genetic Corn Seed, Green: Albino (#17730)
  - o Carolina Biological's Sorghum Seed, Red:Green (#178080)
  - I recommend using two or three distinct seed sources, so that each group may or may not be growing the seeds from the same source as neighboring groups
  - One of the seed sources may be a wild type of the same species for which an F2 cross will be used (advanced option)
  - One of the seed sources may involve a dihybrid cross with or without epigenetic traits (advanced option)
- Instructor lab preparation notebook to record seed source blinding
- Sunny shelf, growing rack with fluorescent lighting, or greenhouse access

### Each group will need:

 One labeled, blinded packet of seeds prepared from one of the above seed sources (about 100 seeds total; a coin envelope or small plastic bag will be suitable)

- One Petri dish (55 mm diameter)
- Three Whatman filter papers or similar (50 mm diameter)
- Bottle of distilled water
- Dissection needle
- Parafilm
- Wax pencil or labeling tape with marker
- Hand lens

### Procedure and general instructions (for instructor):

### Course Context:

This exercise is ideal for the early weeks of a genetics course or for a genetics module in a general biology course. Students should have already been introduced to Mendelian genetics so that they are familiar with relevant terminology (e.g., genotype vs. phenotype; P, F1, and F2 generations; monohybrid crosses). Extensions on these principles (e.g., dihybrid cross, codominance and incomplete dominance, Punnett squares, and Chi-squared analyses) can be taught during the lecture portion of class before this activity begins or while the lab is in progress, depending on whether any related optional or advanced activities will take place.

### Preparations Before Lab:

Prepare one seed packet per lab group as follows:

- For each group, place about 100 seeds from a single seed source into a small plastic bag or coin envelope.
- Mark the packet with a code (e.g., A) before moving on to the next packet.
- Every group's packet may be marked with a distinct code (e.g., A, B, C, D, E...); alternatively, each group with the same seed source could have the same code marked on the packets (e.g., A, A, B, A, B...). The second approach can allow different groups working with the same seed source to compare results after drawing conclusions (see **Student Data** for an example of why this is may be pedagogically valuable).
- Record in the lab preparation notebook the seed source that correlates with each code.

Experimental materials can be collected for each group as described in the **List of Materials** above. The classroom growing space should be sufficient for all groups to place their Petri dishes unstacked on a well-lit surface at or above 20°C.

# Introduction and Hypothesis Development:

The lab is introduced to the class as an inquiry-based introduction to the scientific thought process. This particular lab is a "structured inquiry" in the sense that the inquiry question and general procedures are pre-assigned, but groups must develop their own hypotheses, make

some judgements in recording data, and draw their own conclusions based on that data (Banchi and Bell 2008). Students are informed that the inquiry question for this lab project is, "How do plants inherit cotyledon color?" (see also **Procedure and general instructions (for students)**).

As an introductory exercise, students can discuss within their groups ideas about how inheritance works to refresh their knowledge of transmission genetics from the non-lab portion of the course. After brief confirmatory instructor feedback, groups can move on to brainstorming as many potential leaf color traits as possible based on what they have previously observed in horticultural or natural settings (as opposed to what colors they speculate might be possible). Optionally, the instructor can supply a few reasonable student predictions with known biological processes that are involved (e.g., anthocyanin production, reduced chlorophyll synthesis).

Next, students will begin the hypothesis development phase, which typically takes about an hour when combined with the previous exercises. Groups should be informed that they will be receiving unknown seeds and some details about these seeds (see the student handout). Groups then work separately to generate a hypothesis about the phenotypic ratios for cotyledon color they would expect to see in their own F2 generation seeds. The hypothesis should include both a prediction and some underlying biological reasoning.

The hypothesis generation step is *intentionally* both guided and based on limited information. It would often require a lucky guess for a group's predictions (and therefore hypothesis) to be fully supported after the data analysis phase. This is intended to help students move away from the desire to get the "correct" answer on the first try and towards the use of the hypothesis as a tool to help identify what new information is learned through observation. The former is a pedagogy to which most students have been thoroughly enculturated, while the latter better represents the practice of scientific thinking. I recommend instructor feedback after students have completed this phase of the project.

### Setting Up the Experiment:

This stage can be carried out immediately after hypothesis generation, although it may also be delayed until after students read and reflect on formative feedback related to their group's hypothesis. Details are provided in the student handout. This portion of the work typically takes about 20 min of class time when groups are sharing some resources.

# Phenotype Observations:

This data collection phase may require parts of 1-3 lab periods, depending on the germination times of the species used. Details are provided in the student handout. Early qualitative observations and group discussions to determine on cotyledon color phenotype categories

typically take less than 20 minutes of class time each day. Final data collection typically takes about 30 minutes of class time.

Analysis of Qualitative and Quantitative Data:

Each group uses its experimental data to assess its initial prediction based on both qualitative analysis and a chi-squared test. Each group should record its work and findings in the project lab notebook. This phase and the Drawing Conclusions phase together typically require about 30-45 minutes; one or both can be carried out immediately after data collection if desired. Additional details are provided in the student handout.

Qualitative analysis focuses on the categories of cotyledon color and their relative abundance. Note that "unexpected" relative abundances can be produced by including a wild type seed line or a seed line with a dominant allele that generates a cotyledon color other than green. The latter can create opportunities for later discussion about different ways mutations influence gene function.

Quantitative analysis is based on the chi-squared test. The "expected" cases are based on the specific numerical ratios the group initially predicted, while the "observed" cases are based on the phenotypic categories and ratios the group actually measured. Undergraduate genetics textbooks often provide a step-by-step procedure and a table for determining  $X^2$  and its associated p-value, respectively (e.g., pp. 101-104 in Hartl 2014). Students often need guidance in recognizing that they should base the calculation of expected seed numbers of each trait on the number of seeds actually phenotyped for cotyledon color, rather than the total number of seeds initially "sown" on the Petri dish. Some students may need a reminder of how many degrees of freedom are involved in the test; this is simply the number of phenotypic categories minus one.

Finally, students usually need guidance is in interpreting the meaning of p-values. This may be the case even where students have completed other statistics or lab-based courses, due to the particular way in which chi-squared analysis is used in genetic inheritance. The statistical (null) hypothesis for the chi-squared test is based directly on the biological prediction: if the p-value is less than 0.05, we have demonstrated that "the ratio *does not* fit" and must test another hypothesis about the inheritance ratio. In contrast, most biological experiments use the statistical hypothesis as a quantitatively testable straw man argument: if the p-value is less than 0.05, we have demonstrated that "a difference *does* exist" and the biological prediction (i.e., reason for carrying out the experiment) is supported. Because of these challenges, I recommend groups work on the quantitative analysis at least partly during lab time. This allows time for students to raise questions within their group and while the instructor is present. Groups can also work outside of class on this phase if additional time is needed. Instructor feedback may be helpful at this phase.

### **Drawing Conclusions:**

After qualitative and quantitative analysis, each group should have enough information to more thoroughly evaluate its initial hypothesis and sources of error. This phase typically requires no more than 45 minutes, including both time for group discussion and developing a summary paragraph describing the group's conclusions. If additional time is available, other activities can be built in at this phase. Some of these are described in **Advanced Options**. I recommend a summative assessment of the lab notebook when this phase is completed.

Once each group has turned in its lab notebook for the project, all groups may check their conclusions against the seed packet "key" to find out the actual type of F2 seed they have grown.

## Procedure and general instructions (for students):

*Note to instructors:* 

A project overview and description of each project phase are shown below. This document represents the way I have typically taught this module using tobacco seeds in a twice-weekly, integrated lecture and lab course. It does not directly address most of the optional or advanced activities, but it is compatible with some of them and does include information for *Developing and Testing New Hypotheses* (see **Advanced options**). I recommend providing students with this set of instructions after the introductory refresher and brainstorming activities. It may be possible to follow up with activities such as *Inferring F1 and P generation Genotypes and Phenotypes from F2 Individuals* as a class or homework exercise after groups turn in their lab notebooks. Note that the instructor will need to replace the [###-###] and [###] in Phase IV with relevant page numbers from a relevant textbook.

# Lab Project: Cotyledon Phenotypes and Genotypes

In this project, we will explore the inquiry question: "How do plants inherit cotyledon color?" using standard methods in transmission genetics.

Today your group will receive a packet of "unknown" seeds. All seeds in the packet are from the F2 generation of the same cross. The two parents (P generation) were each true-breeding for some cotyledon color, although the two P generation individuals may have had different cotyledon colors.

The expected project timeline is shown in the table below. The phases are described in more detail afterwards.

Tuesday	Thursday
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	[Lab work]	Introduction, Phase I, Phase II	
Week A	[For next class]	Turn in lab notebook for instructor review of hypothesis	
Week B	Reflect on instructor feedback, refine hypothesis if needed, water seeds if needed, Phase III (qualitative) if possible	Water seeds if needed, Phase III (qualitative)	
	N/A	N/A	
Week C	Phase III, Phase IV; Phase V if null hypothesis is rejected	Phase V, Phase VI, and Wrap-Up	
	Complete chi-squared testing and interpretation	Turn in lab notebook for instructor review of project work and findings	

<u>Phase I – Hypothesis Development:</u> Work within your group to generate a hypothesis about what sort of phenotypic ratios for cotyledon color you would expect to see in your seeds. The hypothesis should include both a *prediction* and some *underlying biological reasoning*.

The *prediction* describes *what* you think you will observe after your seeds have germinated. What cotyledon colors will you see? In what ratios do you expect to see them?

The *biological reasoning* is an explanation of *why* you think you will observe the things you have predicted you will see. For example, how many genes do you think are involved in the inheritance of cotyledon color? For any given gene, how many alleles do you think might exist? What particular pigments are affected by these genes or alleles?

Record your group's thought process and hypothesis in your lab notebook.

<u>Phase II – Setting Up the Experiment:</u> The goal of this step is to create an environment where your seeds can germinate and cotyledon development can occur.

- Place the three filter papers into the base of the Petri dish.
- Soak the papers with distilled water. No excess water should be present in the dish.
- Transfer your seeds from the package into the Petri dish.
- With a fingertip or dissection needle, distribute your seeds evenly across the wet filter paper.
- Cap the Petri dish and wrap it with Parafilm to maintain high humidity in the dish.

- Label you group's Petri dish with a wax pencil or labeling tape and marker so that you can identify it later, even if it gets moved.
- Move the sealed dish to the growing area.

<u>Phase III – Phenotype Observations:</u> During early germination, few seeds will have visible cotyledons. You may take *qualitative* notes at this time and should discuss with your group members how many leaf color phenotypes are present and what those phenotypes are. If helpful, use a hand lens to make judgments about which colors are the same and which are different. You should record your decisions on color phenotypes in the lab notebook, but you do not need to take *quantitative* data until over half of the seeds have germinated.

When most seeds have germinated, your group should record quantitative data. First, identify the distinct cotyledon color phenotypes, then count the number of seedlings with each phenotype. Again, a hand lens can be helpful. You may also want to record the number of seedlings that have died due to drought or infection and the number that have not yet germinated on the day of data collection. These "no cotyledon color recorded" categories can be helpful for thinking about data quality and potential sources of experimental error.

<u>Phase IV – Analysis of Qualitative and Quantitative Data:</u> The goal of this phase is to use your experimental data to assess your group's initial prediction. As before, you should record your group's thinking in your lab notebook.

Qualitative analysis: Did you see the cotyledon color traits you predicted? If not, what other traits did you see instead? You may also want to think about whether particular leaf traits were more abundant or less so than you predicted, without necessarily thinking about specific numbers or ratios.

Quantitative analysis: Use a chi-squared test to determine if your group's data are consistent with the ratios you predicted. The procedure for carrying out this test is provided in pages [###-###] of your textbook, and the table for determining the p-value from the  $X^2$  value and degrees of freedom is found on page [###]. This test involves multiple steps, so feel free to ask questions if you get stuck along the way!

<u>Phase V – Revising the Hypothesis:</u> When an initial hypothesis does not align well with observations, it's time to develop and test a new hypothesis! If your group's seeds had different cotyledon colors than you expected, or if those seeds showed up in different relative proportions than you expected, or if you rejected your null hypothesis in chi-squared analysis, spend some time as a group revisiting Phase I. This time, you come to hypothesis development with much more information about your seeds than you had earlier. Feel free to use those observations to rethink both the predicted inheritance ratio and the underlying biological process involved.

Once you have created a revised hypothesis (prediction + underlying biological reasoning), revisit Phase IV to show how your prediction aligns with the qualitative data you have. Carry out a new chi-squared test to determine whether or not you can reject the new null hypothesis.

<u>Phase VI – Drawing Conclusions:</u> The goal of this phase is to re-evaluate whether and how your ideas about cotyledon color inheritance have changed through the course of the experiment. Discuss the following questions with your group members:

- Do you think your data are reliable enough to evaluate your initial hypothesis?
- How did you decide whether or not your data were reliable?
- What possible sources of error do you think might have influenced your experiment?
- If you repeated this experiment to improve data quality, what factor(s) would you change and why?
- Were your qualitative and quantitative results consistent with your group's initial prediction? Why or why not?
- What are the implications for your group's initial biological reasoning?
- Was any alternative hypothesis more compatible with your observations? Why do you think so?
- How has your thinking changed as you moved through the experimental process?

You may want to take notes on your group discussion, but it is not absolutely necessary. When you've completed the discussion, write a paragraph to summarize your answers to these questions. Once you turn in your lab notebook, I will post the seed packet key so you can validate your results.

#### Suggestions for assessing student learning:

I typically require that all groups turn in a lab notebook documenting the group's thought processes and initial experimental setup (i.e., after completion of Phases I and II). Providing formative feedback on each group's initial hypothesis and level of procedural detail can be valuable early in the course so that students familiarize themselves with any lab notebook grading rubric and to help the instructor assess students' comfort level with hypothesis development in a lower-stakes situation.

I base summative assessment of the project on the lab notebook after the completion of the conclusions phase. One possible lab notebook grading rubric might evenly weight the following components:

- scientific thought process (including hypothesis development and refinement, interpretation of p-values, and evaluation of the hypothesis),
- documenting procedures (including deviations from any standard protocols),

(<a href="http://lifediscoveryed.org/">http://lifediscoveryed.org/</a>)

- documenting data (including quantitative units and definitions of subjective categories),
- documenting calculations and data analysis, and
- evidence of equitable participation.

#### Student data:

Asking students to make their own judgements about seedling phenotypes can potentially have rich pedagogical benefits. When teaching this module with tobacco green:yellow green:yellow 1:2:1 seed, I have noticed that student groups differ in their tendencies to "lump" or "split" ambiguous phenotypes such as yellow green and green. For example, during an upper-level Genetics course, one group that received these seeds analyzed only two distinct categories despite recording three categories during their observation phase as follows:

Named Phenotype	Dark Green	Light Green	Yellow
Initial Observations	5	21	11
For Chi-Squared	26		11

This presents a "teachable moment" related to the difficulty scientists may face in recording qualitative data, to changes that occur during seedling development, and to the similarities and differences between genotypes and phenotypes between two modes of inheritance (Mendelian dominance and incomplete dominance). Allowing students to compare their observational categories and analytical strategies across groups after each group draws its conclusions is one way of highlighting these ideas for the whole class.

### Advanced options:

Developing and Testing New Hypotheses:

Due to the relatively broad nature of the hypothesis generation phase, some groups may reject their null hypothesis during qualitative and/or chi-squared analysis. More engaged students may be left with a lack of closure: "if the hypothesis was incorrect, what was the *real* inheritance ratio?" Furthermore, less engaged students that fail to reject the null hypothesis during chi-squared analysis may be left with an overly confident sense of the certainty of their hypothesis: "my answer was the *real* one, I do not need to consider alternative hypotheses."

One way to further engage both types of students is to require that groups develop and use the chi-squared method to test at least one new hypothesis after completing the initial one. Groups that reject their initial hypothesis are able to engage in a realistic hypothesis refinement process. These students will experience the iterative nature of science in the sense that they need not start "from scratch" in their thinking, but can instead base their new predicted inheritance ratios on actual observations. Alternatively, testing secondary hypotheses can

highlight the need to consider multiple hypotheses before settling on a single explanation as the correct one, particularly in cases where more than one hypothesis cannot be rejected (e.g., epistatic traits may sometimes be difficult to distinguish from characters determined by incomplete dominance or single-locus dominance and recessiveness).

In cases where multiple hypotheses about inheritance cannot be rejected, the entire class can gain a sense of closure by designing (but not necessarily carrying out) one or more experimental crosses. By (hypothetically) growing the F2 seeds to maturity, plants with specific cotyledon colors can be selected for crossing to distinguish between the two hypotheses.

Inferring Multigenerational Genotypes and Phenotypes from F2 Individuals:

An optional activity to conclude the project, and one that will likely provide a direct connection to transmission genetics homework problems, is to ask each group to infer the possible genotypes of its F2 seeds of each phenotype. The most straightforward approach in this case is to allow groups to first compare their conclusions about their F2 seeds' inheritance patterns against the "key." With greater certainty about the phenotypic ratio, the group can directly determine the F2 genotypes of their seeds.

This activity can be further elaborated by encouraging students to determine the genotypes and phenotypes of the two previous generations of seed. Since students have been informed that the P generation plants are both true-breeding, determining the F1 and P generation genotypes should be compatible with textbook and homework problems if cotyledon color is determined by a single locus. Once genotypes are assigned, determining phenotypes should also be straightforward: all possible phenotypes would be visible (and already associated with specific genotypes) in the F2 seeds.

This task becomes more complex for phenotypes involving two loci (see also *Dihybrid Crosses or Epistatic Traits*). In this case, it would be more valuable to have students consider the *possible* P generation genotypes and phenotypes instead of focusing on getting a single "correct" set.

While the above approach is straightforward, scientific realism can be added in classes where students have strong grounding in scientific thinking skills (e.g., an upper level Genetics course for biology majors). In this case, each group can determine the F2 genotypes based exclusively on the group's own experimental conclusions, without reference to the "key." Determination of F1 and P generation genotypes and phenotypes can then be carried out as previously. This approach allows students to deduce new information without reference to any new external information, but technical or logical errors made during analysis or while drawing conclusions can be propagated at this stage. For this reason, any summative assessment of this work should be structured to avoid penalizing students multiple times for an early error.

Addressing the Possibility of Lethal Alleles:

If students have learned about genetic lethality prior to the hypothesis generation phase, it may be helpful to tell students that seeds may not germinate, or may die after germination, due to overwatering, underwatering, fungal or bacterial growth, or other factors. Lethal effects in seeds most often occur during seed development, prior to germination; as long as the seeds do not appear to be "deflated" or off-color when they are first placed on the Petri dish, it is fairly unlikely that lethal alleles are involved. Eliminating lethal genetic effects as a possibility can help students focus on other possible explanations for the observed phenotypic ratios.

## *Including Wild-Type Seeds:*

One way to guarantee unexpected F2 results is to include one or more packets of wild-type seeds in the class. Ultimately, the knowledge that both individuals in the P generation were true-breeding should help most students narrow down the possibilities to focus on two homozygous wild-type parents. One exception is that introducing this module after teaching about epistasis and/or complementation analysis allows an epistatic interpretation of F2 seeds that all share the same phenotype.

I have included wild type seeds for at least one group every time I have taught this lab project. The primary challenge for the groups that receive these seeds is confusion over how to work with predicted phenotype categories that had no observations in the chi-squared analysis. Once students are reminded that they *expected* a non-zero number of seeds in these categories and *observed* zero seeds with such phenotypes, they are able to complete the analysis successfully.

# Dihybrid Crosses or Epistatic Traits:

If students have already learned about dihybrid crosses at the hypothesis generation stage, it would be reasonable to include at least one set of F2 seeds that show variation in a second character in addition to cotyledon color. In this case, asking that groups take notes on their seeds beyond just cotyledon color will help demonstrate the scientific value of making broad observations, rather than observations exclusively focused on the hypothesis. After completing chi-squared analysis based only on cotyledon color traits, groups could be asked to refine the initial hypothesis to incorporate phenotypes for the second character. Testing this hypothesis would involve a more comprehensive chi-squared analysis.

Alternatively, if students learn about two-gene epistasis prior to or during this project, it would be reasonable to include at least one set of F2 seeds exhibiting epigenetic variation in cotyledon color. Note that relatively low (<100) seed numbers may not be sufficient to reliably differentiate between epigenetic inheritance and dominant/recessive or incomplete dominance inheritance patterns using a chi-squared test. This creates a clear opportunity where a second chi-squared analysis could be valuable even if the initial analysis was unable to reject the null hypothesis (see *Developing and Testing New Hypotheses*).

# **Reference list**

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