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Using *C. elegans* to Test the Adaptive Significance of Sexual Outcrossing

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Few laboratory exercises focus on the adaptive significance of sexual reproduction. The investigation described here involves students in a test of one of the predictions of The Red Queen hypothesis—namely, that sexual outcrossing is superior to self-fertilization. Specifically, students challenge two populations of the nematode *C. elegans*—one obligately outcrossing and one obligately selfing—with pathogenic *Serratia marcescens*.

Keywords: evolution of sex, sexual outcrossing, The Red Queen, *C. elegans*

Introduction

In response to demands for improved biology education for all students, I created the Evolution and Biology of Sex—an introductory-biology course for non-biology majors. This course, which is currently in its twelfth year, enrolls ~860 students each year and uses evidence-based educational principles in both large-lecture and laboratory settings (Cotner, 2017). The course is accompanied by a semester-long (fourteen-week) laboratory component, in which students work in small (4-person) groups—for two hours each week—to delve deeper into topics such as sexual selection, the adaptive significance of sexual outcrossing, sexually-transmitted-disease transmission, human population growth, and human sperm competition. Many of these exercises have been discussed elsewhere (Cotner and Wassenberg, 2018; Ballen, Thompson, Blum, Newstrom, & Cotner, 2018; Cotner & Gallup, 2011; Cotner & Hebert, 2016), so I have selected to present one innovative investigation—Using *C. elegans* to test the adaptive significance of sexual outcrossing. This exercise was modeled after a paper by Levi Morran and colleagues (Morran, Schmidt, Gelarden, Parrish, & Lively, 2011).

The Red Queen and the Evolution of Sex

In evolutionary biology, a fundamental question centers on Why Sex? Specifically, given the significant ecological and genetic costs of sexual reproduction, how can we understand the evolution and maintenance of sex in so many different taxa? While several hypotheses have been advanced to explain the evolution of sex, our discussion focuses on The Red Queen (Ridley, 1993). According to The Red Queen hypothesis, sex exists as a way for organisms to keep up with rapidly co-evolving pathogens. Sex allows the generation of genetic diversity in lineages, and makes pathogen hosts a moving target. Like Alice and the Red Queen in Lewis Carroll's novel (*Through the Looking Glass*), both host and parasite are running a race in which neither makes any observable progress. Yet, if the host organisms don't change dramatically with each new generation (via meiosis and outcrossing—that is, if they don't have sex), the lineage might go extinct.

In sum, by generating genetic diversity at a pace that exceeds that of asexual organisms, sexual organisms are better able to combat their rapidly evolving parasites and pathogens.

Testing The Red Queen

The Red Queen is a hypothesis that can be broken down into several predictions. One of these predictions—sexual outcrossing is superior to self-fertilization (i.e., outcrossing individuals have greater fitness than their self-fertilizing counterparts)—is the focus of this laboratory investigation.

Laboratory Overview

Students will investigate The Red Queen hypothesis by observing the interaction between the nematode *Caenorhabditis elegans* and its pathogenic bacteria *Serratia marcescens*. Specifically, they will test one prediction of The Red Queen: sexual outcrossing is superior to sexual selfing, rendering the progeny of outcrossing nematodes better able to combat pathogens.

Student Outline

Objectives

- Describe The Red Queen hypothesis for the evolution and maintenance of sexual reproduction
- Describe the life-history characteristics of *C. elegans*
- Evaluate the pooled class data in light of one specific prediction (did you gather evidence that suggests that sexual outcrossing can be superior to selfing?)

Introduction

Who is C. elegans?

C. elegans is a small, transparent roundworm that lives primarily in soil. The animal has been a model organism for many areas of research from disease research to space expeditions. The nematode is reproductively mature in about 3 days at 20 °C and lives between 2–3 weeks. The worms we'll investigate primarily eat *E. coli* but can live up to three months with no food at all. (Because the agar in your Petri dish is nutrient-rich, keep the lid on as much as possible to prevent unwanted microbes from colonizing your dish.) The worms are constantly foraging and consuming whatever they are moving through; if *S. marcescens* is present, the worms will eat it. It can take as little as 24 hours from the time of consuming the live *S. marcescens* to death by infection.

With *Caenorhabditis elegans*, the populations in nature exist in two sexes, males (XO) and hermaphrodites (XX). When males are present, hermaphrodites outcross with them. If you observe two worms continuously sliding past each other, this is probably a male and a hermaphrodite having sex. When no males are present, hermaphrodites, who have both gametes, will lay self-fertilized eggs. Hermaphrodite *C. elegans* have not been observed cross- fertilizing with other hermaphrodites. The two populations you will be working with have been genetically altered from the wild-type (male and hermaphrodite) into obligately selfing (hermaphrodite) and obligately outcrossing (male and female) worms.

Specifically, the worms have been bred to have one of two known mutations: the *xol-1* (XO lethal) and *fog-2* (feminization of germination). The xol-1 mutation results in reduced expression of the X chromosomes; the effect is lethal in males (who possess only one X chromosome), leaving an entirely selfing population of hermaphroditic worms. The *fog-2* mutation disables sperm production in hermaphrodites generating "female" *C. elegans*, therefore the wildtype (male and hermaphrodite) population becomes an entirely outcrossing (male and female) population. The original strain CB4856 was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN).

Testing The Red Queen

You will work with two different populations of nematodes, group X and group Y, and their coevolved strain of *S. marcescens*. You will conduct a *single-blind experiment*, meaning that you will not know which of the two groups of worms you will be observing, but your TA will (in a double-blind experiment, neither you nor your TA would know which group of worms you are observing). You will be exposing the worms to *S. marcescens* that has been co-evolving with worms for 30 generations. Make sure that you keep the "X" strain of *S. marcescens* with the "X" strain of worms!

For week one you will place *S. marcescens* into your dish. You will also add some *E. coli* for food. In week two, you will transfer ~15 worms from a growing stock population to your dish. In week two you will count worms (that were previously added to dishes prepared with *E. coli* and *S. marcescens*), alive and dead, and calculate mortality (as a percent of total worms) for your population of nematodes. You will also transfer ~15 worms from a growing stock population to a prepared dish (this is for students in another class). You will combine your data with that of the other students in your lab section, and with the class as a whole. This should allow us sufficient replicates to safely support or reject our hypothesis.

Some of the procedures (inoculating plates with *S. marcescens*, transferring worms) are illustrated in a short video at: https://www.youtube.com/watch?v=LJFsJT4rv4Q (note that the procedures described here differ in some respects).

Pre-Lab Questions

1. Explain, using your own words, what The Red Queen Hypothesis is.
2. Place the outcome of these reproductive modes in order of whether they generate the greatest diversity (1) to least diversity (3).

___ Sexual selfing

___ Asexual reproduction

___ Sexual outcrossing

3. What is outcrossing and how is this beneficial?
4. How is it possible to have sex without outcrossing?
5. What are two problems that outcrossing organisms face?
6. What is meant by the "cost of males"?
7. What is an "evolutionary arms race"? Give a few examples.
8. In which ways can *C. elegans* reproduce? Explain.
9. What do you expect to see happen to Group X and Group Y after one week? Explain your answer in the context of The Red Queen.

Methods and Data Collection

Lab One. Preparing the plates

Materials

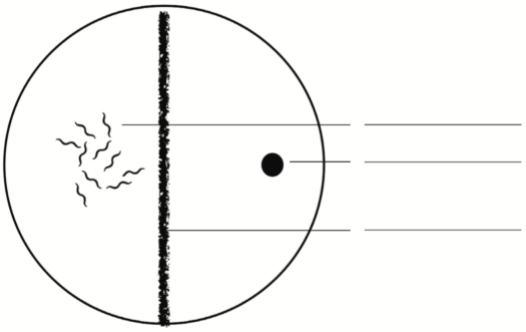
- micropipette
- 10 μm *S. marcescens*
- *E. coli*
- 1 Nematode Growth Medium (NGM) Petri dish

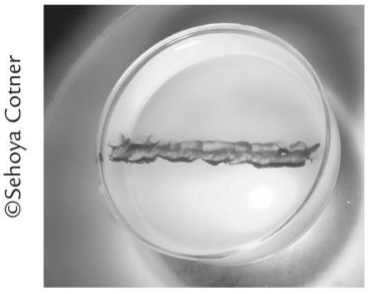
Plating Procedures: Turn your agar plate over (with medium facing up), and use a permanent marker (e.g., a fine-tip Sharpie) to mark the bottom with a small dot. This is where you'll drop your *E. coli*.

Micropipette 10 μm of *S. marcescens* from your cultured tube. Touch the tip to the agar (being careful not to puncture the agar) and drag back as you dispense the culture onto your NGM Petri dish in a straight line across the center, thus dividing the dish into two halves. After all 10 μm are dispensed you can go back along the line to complete any breaks in the bacterial line. Note: The color of the liquid *S. marcescens* in your tube should be pink/red but 10 μm across the dish should look almost clear. The bacteria will grow and next week you should find a dark-red culture in your dish.

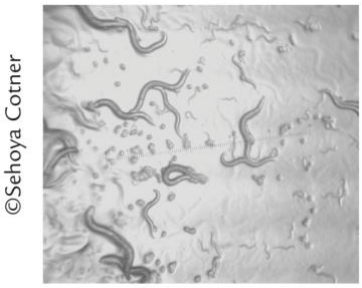
Micropipette 10 μm of *E. coli* and place in a single drop on the side of the plate labeled (from below) with a dot.

To illustrate your understanding of how these plates were prepared, please label the following parts of the figure, below left. Your instructor will check your understanding.





Cultured *S. marcescens*



C. elegans at 40X

Lab Two: Work Picking and Transferring

Seed 15 nematodes on one side of a prepared dish (away from where you placed the *E. coli* in week one). [Optional: Students in a different lab section may collect mortality data from this population of nematodes.]

Materials

- Your NGM dish prepared in week one (with *E. coli* and *S. marcescens*)
- Stock Nematode dish
- Toothpick

To pick worms, touch a toothpick to the agar. Depending on how dense and how healthy the worms are you may pick up 0 worms, or you may pick up 50. For a more accurate count, try to find an area where the worms are more dispersed. Picking a few worms at a time is safer than trying to pick up a whole group. As soon as you touch your toothpick to the agar in the new dish the worms will begin to crawl away. Count as fast as you can because they crawl quickly.

1. Bring the dissecting scope to the edge of the table.
2. Turn "front (or top) light" off.
3. Turn "back (or bottom) light" on.
   Careful, Nematodes Burn!
4. Use the lowest power (objective lens) for your initial focusing.
5. Find the surface of the agar in your dish.
6. Use the highest power to count your nematodes.

IMPORTANT: Using too much light heats up the worms and agar, so adjust your light so that you can see the worms, but don't have to squint because the field of view is too bright. Within 2 minutes of turning on the light, nematodes stop moving. If this happens, they may not recuperate. Keep your light off whenever possible.

Rest your picking hand on the base; this helps you:

- avoid breaking through the surface of the agar
- locate your toothpick under the dissecting scope

Watch under the scope as you place each worm on the new Petri dish to verify that it has been transferred. Try to transfer ~15 worms (no fewer than 10!); transferring many more than 15 is not as much of a problem as transferring many fewer. Can you imagine why this is the case?

Label your dish with your population of nematodes (X or Y), your name, and your section number.

Lab Three: Collecting Mortality Data

Overview

Today you will count the number of living nematodes and dead nematodes, and then calculate a percentage of dead nematodes to obtain "percent mortality." Your instructor will guide you in this step. [Optional: you may be counting nematodes that were prepared by students in a different lab section, just as you may prepare nematodes for somebody else to count.]

Real-time Counting

Have your data-collection sheet and a pencil next to you. Set the dissecting scope on the highest objective power. Everything you can see through the lens is one "field of view." You should count the worms in 5 fields of view.

Count all the worms you see as you move your dish back and forth left and right and from top to bottom. You may find it helpful to mentally subdivide the field of view into quarters, using the scale bar (if there is one) in your field. Mark the number of worms in the first cell of the "Total Nematodes" column. Next, go back through the worms, only counting those that are dead (your TA will help you identify dead worms; they are not moving, may be "frozen" in an unnatural pose, and may be clearly ruptured). Mark the number of dead worms in the first cell of the "Dead Nematodes" column. You can fill in the "Live Nematodes" column by subtraction, although you can also just leave this column blank — it is not necessary for counting percent mortality.

Two individuals should count everything independently, and average these two counts for a better approximation. If you and your partner come up with wildly different numbers, you should take time to ensure that you are using similar methods.

Repeat this counting and tallying technique for four more fields of view. Disregard the dark opaque strip of *S. marcescens* across the middle if no light passes through; there may be a few within this area but it's too difficult to see them. Depending on skill level, counting five fields of view should take 15-20 minutes.

On your data sheet write down the number of total nematodes for each field of view as well as dead nematodes for each field of view. Calculate mortality. Pool your data into the class results and construct a graph.

Live Nematodes			Dead Nematodes			Estimated Total
Student 1	Student 2	Average	Student 1	Student 2	Average	#living worms + #dead worms

% living:_____ % mortality:_____

Was your sample from the selfing or outcrossing population of *C. elegans*?

POOLED CLASS DATA:

% Mortality, Selfing	% Mortality, Outcrossing

Are these percentages different? If so, are the differences statistically significant? (Your instructor will help with tests of significance, if warranted.)

Post-Lab Questions

1. Return to pre-lab question #9. (What did you expect to see happen to Group X and Group Y after one week?) Did your results meet your expectations? If so, what do these data mean? If not, what conclusions can your make regarding the adaptive significance of outcrossing?
2. How did your group's data compare with the class data? If there are differences, how can we understand these differences?
3. Graph your data. Be sure to label your axes.

Materials

The following materials are necessary, and their use is described in the Student material and in the Appendices:

To make Nematode Growth Medium (NGM) plates*:*
- 3g NaCl
- 17g Agar
- 2.5g peptone
- 975mL water
- 1 mL 1M $CaCl_2$
- 1 mL 5mg/mL cholesterol in ethanol
- 1 mL 1M $MgSO_4$
- 25 mL 1M KPO_4 buffer

100 ml petri plates
35 ml petri plates
Cotton swabs
Sharpie™ markers
Micropipettes
Gloves
Parafilm
Dissecting scope
C. elegans (mutants described here are available from the Caenorhabditis Genetics Center*:* https://cgc.umn.edu/)
S. marcescens
E. coli OP 50

The appendices contain additional information:
- Appendix A. Making NGM plates
- Appendix B. Preparing student plates
- Appendix C. Student instructions for plate preparation

Notes for the Instructor

This lab is currently run in stages over the course of one week, rather than using the three-week (or three-lab) schedule described here. With the condensed option, the initial plates (with *Serratia* and *E. coli*) are prepared prior to lab, then each lab section transfers nematodes to plates *and* counts living and dead nematodes on another set of plates prepared by a different lab section.
It is not unusual for some lab sections to collect data that are inconclusive. By pooling the data from several sections, we always find compelling evidence that sexual outcrossing is adaptive in the face of the pathogen *Serratia.* Sample data are available at: https://drive.google.com/file/d/1JG7uwK9jSY38Z8KOAPbHFjn0mOODDVfD/view

For a thorough guide to working with nematodes, consult: www.wormbook.org

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About the Authors

Sehoya Cotner is an Associate Professor in the Department of Biology Teaching and Learning at the University of Minnesota-Twin Cities. Sehoya has been teaching The Evolution and Biology of Sex for 12 years—living the dream!

Appendix A: NGM Stock Agar Plates

Mix the following in a 2L flask:

- 3g NaCl
- 17g Agar
- 2.5g peptone
- 975mL water

Cover with tin foil and autoclave on liquid
Cool flask in a 55$_{o}$C water bath for 15 minutes

Add:

- 1 mL 1M CaCl2
- 1 mL 5mg/mL cholesterol in ethanol
- 1 mL 1M MgSO4
- 25 mL 1M kPO4 buffer

Swirl to mix
Pour 100mm plates 2/3 full of agar
Leave at room temp for 2-3 days- dispose of contaminated plates
Store in an airtight container at room temp for several weeks.
(for class work use 35mm plates)

Appendix B: Making Student Nematode Plates

Using a fine tip Sarpie™:
1) On the bottom of the plate (agar side) draw a line bisecting the plate into two halves.
2) Draw an "x" on one side of the plate.

Using a cotton swab dipped in *S. marcescens*

3) Draw a line following the Sharpie™ line

Notes:

- can use the same swab multiple times ~10 plates
- dispose swabs into a discard beaker lined with autoclave bag

Using a micropipette and the *E. coli* OP 50 culture

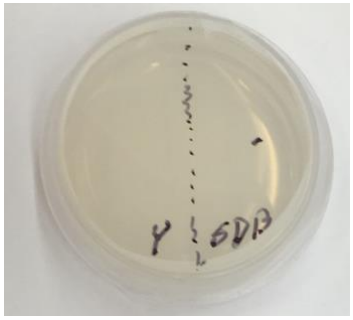
4) Add 10µL to the side with the "x."

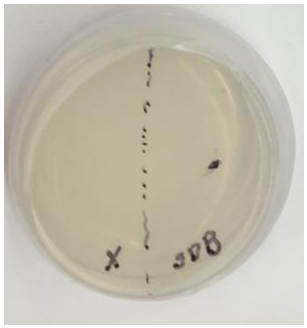
Notes:

- can use the same tip for ~10 plates
- make sure the *E. coli* OP 50 is not in contact with the line
- dispose tips into a discard beaker lined with autoclave bag

Appendix C: Instructions to Prep Nematode Plates

1) Put on gloves and goggles. Then obtain a plate.
2) Use a Sharpie™ to make a line, a dot to one side of the line, and your initials on the **bottom** of the plate. (The bottom of the plate is where the agar is located)
3) Place the cotton swab into the tube of *S. marcescens*. Use the cotton swab to lightly draw a line of *S. marcescens* across the agar to divide it in half. This line will be a faint pink, but by next week will be a darker red.
(Side note: *S. marcescens* is fatal to most nematodes and you are creating a barrier the nematodes must cross to get to the food source.)
4) Discard the cotton swab into the discard beaker.
5) Using the micropipette with a clean tip, draw up 10 □L of *E.coli OP50*. Put all of it in one spot, where you marked the dot. Discard the tip into the discard beaker.
Side note: this is food for nematodes.
6) Wrap your plate in parafilm along the outer edge.
7) Use a sharpie to mark and mark the plate with an **X** if you used *S. marcescens* X or mark the plate with a **Y** if you used *S. marcescens* Y.





Your plate should look like one of these when you are done. Place in the appropriate tin tray on the back counter.

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