

A Completely Hands-On Paternity Dispute Using DNA Markers and Rapid-Cycling *Brassica rapa* (Fast Plants Type)

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Students resolve a paternity dispute resulting when a female has mated with two males and produced a child. The project uses rapid cycling *Brassica rapa* (Fast Plants) so it can be fully hands-on for the students. Students pollinate a plant (Mother) with a mixture of pollen from two other plants (Alleged Fathers) to produce the Child. Students purify DNA from leaf tissue and then use PCR and gel electrophoresis to determine genotypes for DNA markers. Multiple child genotypes are possible because one of the Alleged Fathers is heterozygous for each marker used while the other Alleged Father is homozygous.

Keywords: Fast Plants, genetics, genetic marker, DNA fingerprinting, paternity test

Link to Supplemental Materials: <http://www.ableweb.org/volumes/vol-34/wendell/supplement.htm>

Introduction

In this lab, students create a paternity dispute using rapid-cycling *Brassica rapa* (RCBr) by pollinating one plant (Mother) with a mixture of pollen from two other plants (Alleged Fathers). The resulting seeds are sown to produce the Child (Fig. 1). Students then use genetic markers to perform paternity exclusion. The markers are DNA-based markers that have alleles that can be clearly resolved on simple agarose gels. The trait-based marker *anthocyaninless*, which affects stem color (dominant purple versus recessive nonpurple), can also be included. This lab is carried out over several weeks, but there are only two times when a full (3-hour) lab period is required. We have used this lab in both introductory level general biology lab and in a sophomore/junior level genetics lab course.

RCBr, also known as Fast Plants, have several qualities that make them well suited to experiments in genetics. They have a very short life cycle, petite growth habit, and have been selected to grow under very simple conditions (Williams and Hill, 1986). For classical genetics, there is variation between strains in Mendelian traits such as stem color (dominant purple versus recessive nonpurple) and leaf color (dominant green versus recessive yellow-green) (Williams, 1985). Molecular tools are now also available. Recently we have developed a group of DNA-based genetic markers for RCBr and their place in the *Brassica rapa* genome

has been characterized (Slankster et al., 2012). Performing genetic crosses with RCBr is made easy by the fact that they are self-incompatible for mating (Musgrave, 2000). Even though they have perfect flowers, a plant is able to detect and reject its own pollen. However a plant can be pollinated by any other RCBr plant, even one of the same strain. To perform a genetic cross you simply use a swab to collect pollen from the anthers on one plant and deposit it on the stigma of another.

We begin the lab by presenting each group of students with three plants: a Mother and two Alleged Fathers. The two Alleged Fathers differ from each other in their genotype for several DNA-based genetic markers (Table 1) and for the classical marker *anthocyaninless* which has the dominant allele *ANL* for purple stems and recessive allele *anl* for nonpurple stems. The students then use one cotton swab to collect pollen from both Alleged Fathers and use it to pollinate the Mother. At this time, they also record the color of the hypocotyl of each plant (either purple or nonpurple) for later scoring of *anthocyaninless* and collect one leaf from each plant for later DNA purification. The Mother plant is then allowed to develop seeds over three weeks, and the Alleged Fathers can be discarded. After the three weeks, the Mother is kept an additional week without watering to allow seed maturation. Seedlings, which are the Children, are then

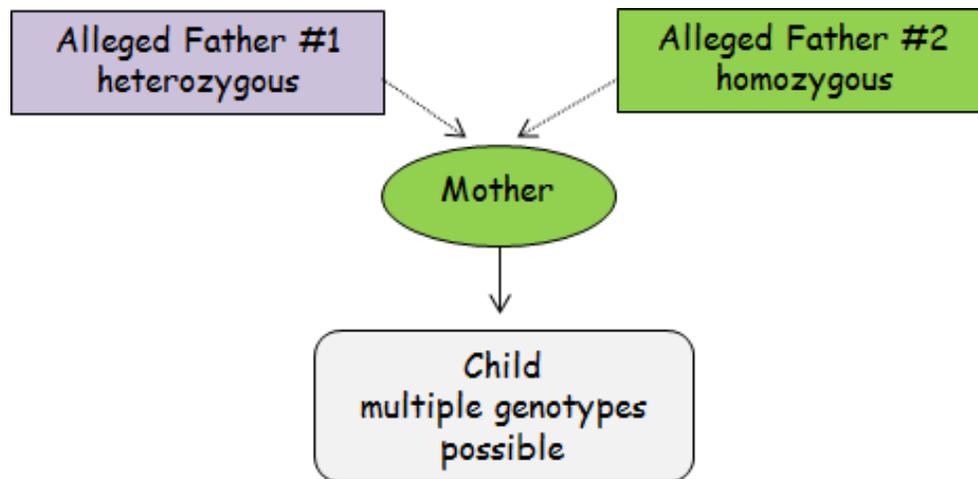


Figure 1. A paternity dispute is created when the Mother is pollinated with a mixture of pollen from two other plants (Alleged Fathers). One Alleged Father is heterozygous so that multiple Child genotypes are possible.

grown either by planting the seeds in potting soil for one week or sprouting them for three days on a wet filter paper under bright light. Students then purify DNA of each Alleged Father, the Mother, and the Child and use this as a template for PCR with any of the DNA markers shown in Table 1. In the final period, the students resolve and detect their PCR products by agarose gel electrophoresis and staining with a SYBR Safe or Gel Green. Students then determine their plants' genotypes for *anthocyaninless* and any of the DNA markers used. Finally, they analyze their data to see which, if any, of the Alleged Fathers can be excluded based on marker genotype.

We have developed two types of DNA markers specifically for this lab. Variable number tandem repeat (VNTR)-type markers are based on variation in the length repetitive DNA sequences. They are detected by polymerase chain reaction (PCR) followed by gel electrophoresis to identify the different alleles by the size of the PCR fragments (Fig. 2A.). The other type of marker is PCR-RFLP markers in which a single nucleotide polymorphism (SNP) resides in a restric-

tion endonuclease recognition site so that one allele is cut by the enzyme and the other is not (Konieczny and Ausubel, 1993). They are detected by PCR followed by digestion with the appropriate enzyme and gel electrophoresis to separate and detect the fragments (Fig. 2B.). Although many DNA markers are available for *Brassica rapa* we developed our markers specifically for teaching lab conditions. They amplify robustly from RCB_r DNA, have alleles that can be resolved in simple agarose gels, and are polymorphic in Fast Plant stocks (Slankster et al., 2012). Sample gel images of all of the markers listed in Table 1 can be found at <http://humangeneticsmustard.blogspot.com/>.

If one of the Alleged Fathers is heterozygous for the markers being used then a variety of Child genotypes are possible. As we currently run the lab, one Alleged Father is heterozygous for all markers including the *anthocyaninless* locus and the other is homozygous. For each used, there are two possible results for the Child genotype.

After students obtain genotype data, they can analyze it methodically. First, they should determine what allele(s)

Table 1. DNA markers for RCB_r.

Marker	Allele	Type	Primers
<i>D1BrapaS1</i>	2	VNTR	GGAGGAGCAAGCAGGACCAGGA ACGCTGTGATTTGTTGCTTCCGA
<i>D9BrapaS1</i>	2	50 bp deletion	CCAGCCAAATCGTCACTCATGCGA TGCATGCCTAAGAGTTTGGAGTAACAC
<i>D9BrapaS4</i>	3	VNTR	AGCGATGTAGCACCCGAGTCCA TCGAGCTGAGAGGGAAGCTGTGA
<i>Park9-HaeIII</i>	2	PCR-RFLP	TCCTCAGCTGCTTTAGCCTC TTGCGACAAAGAAACACAGC
<i>Park14-EcoRI</i>	2	PCR-RFLP	TGTGCTGTAAGTCAAAGCA CGCAAATCACGAGTTCTTCA

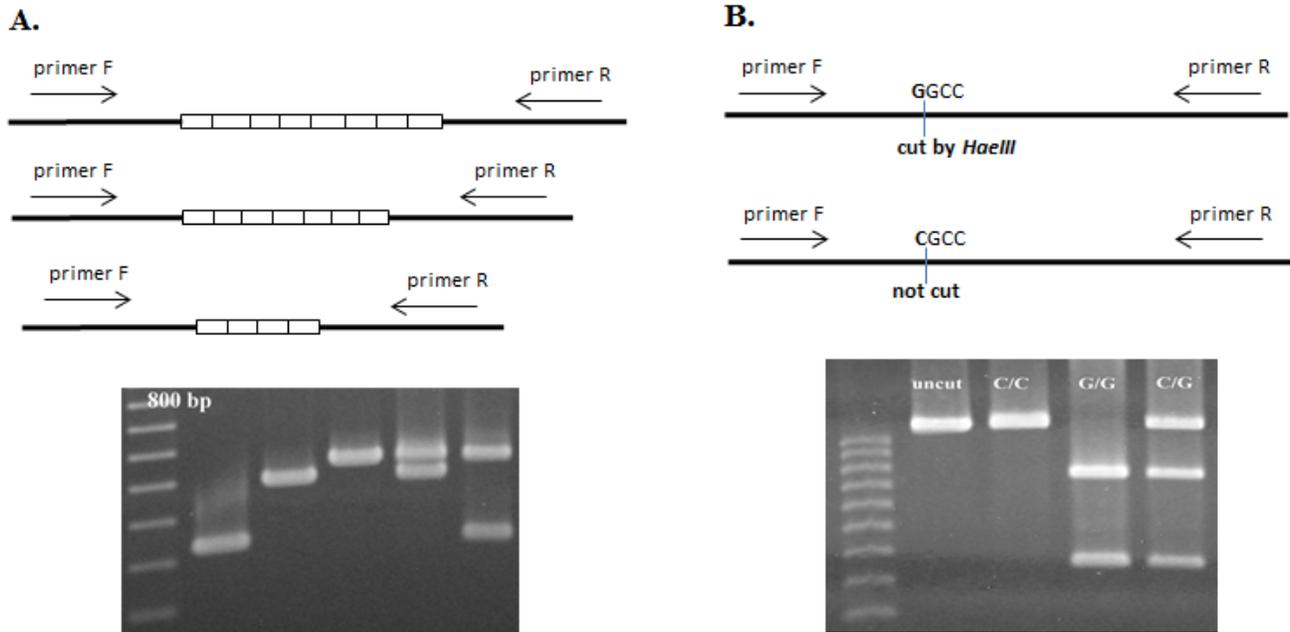


Figure 2. DNA markers for RCB. (A.) VNTR-type based on variation in a repetitive DNA element. (B.) PCR-RFLP based on a single nucleotide polymorphism.

the Child could have inherited from its Mother. In the cases shown in Figure 3A., the marker has two alleles. If we call the lower band *allele1* and the upper band *allele2* then the Child is heterozygous and its mother is homozygous for *allele2*. Therefore, one can deduce that the Child inherited *allele2* from its Mother. Next, the students should deduce what allele the Child inherited from its father, whoever that is. In the cases shown in Figure 3A., the Child's *allele1* must have come from its father. Finally, the genotypes of the Alleged Fathers can be examined to see if either can be excluded. Alleged Father #2 can be excluded because he lacks *allele1*. In the case shown in Figure 3B., the Child is homozygous for *allele2* so it must have inherited one copy from the Mother and one copy from its father, whoever that is. Both Alleged Fathers have *allele2* so neither can be excluded. A case where neither father can be excluded should not be viewed as failure. The students must use their analytical skills to realize that neither can be excluded.

If you use multiple markers, the number of possible Child genotypes increases and the chances that a dispute will be informative for at least one of them will increase. If you incorporate the *anthocyaninless* marker into the lab, your students can get additional genetic data without the additional cost and time needed to genotype another DNA marker. Suppose that in the cases shown in Figure 3, Alleged Father #1 was purple (the dominant trait) and Alleged Father #2 and Mother were nonpurple (the recessive trait). If a child is purple, then Alleged Father #2 can be excluded, but if the child is nonpurple, then neither Alleged Father can be excluded (because Alleged

Father #1 is heterozygous). Using just one DNA marker and the *anthocyaninless* marker, there are four possible Child genotypes.

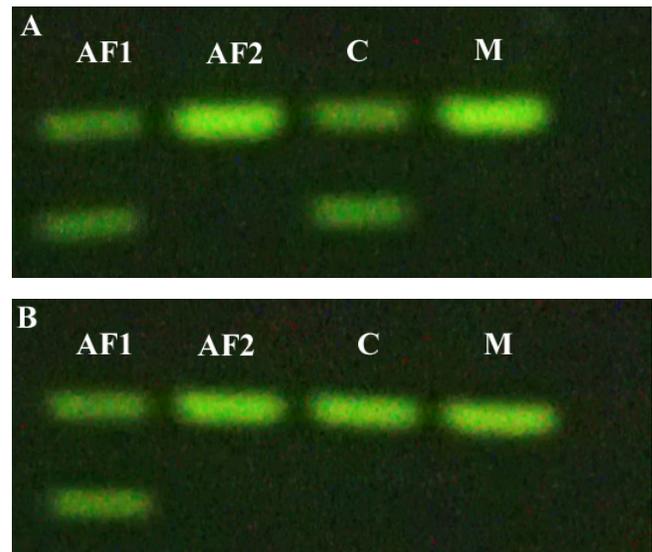


Figure 3. Possible outcomes for one marker when Alleged Father #1 (AF1) is heterozygous and Alleged Father #2 (AF2) is homozygous. The images of agarose gels stained with SYBR Safe DNA Stain were taken using a camera phone. C and M are the Child and Mother respectively.

Student Outline

The following is an outline of the tasks performed by the students. A complete lab manual with protocols and explanatory text is available for download at https://files.oakland.edu/users/wendell/web/Teaching_resources.html. There you will also find an instructor's reference that lists the materials to prepare for each week of the lab.

Lab Timeline

Week One: Create the paternity dispute

- Learn the anatomy of the plant (a link to diagrams is in the lab manual) and compare them to the plants in front of you.
- Use a cotton swab to collect pollen from each of the Alleged Fathers and then touch the swab to the stigma of the Mother plant's flowers.
- Examine the color of the hypocotyl of the plants and record each plant's phenotype (purple or nonpurple).
- Collect one leaf from each plant and store it in a 1.5 mL microtube that is labeled by the plant's ID number.

Weeks Two and Three: Seeds are developing

Week Four: Remove the Mother from the water wick

- Four days from now, your instructor will collect seeds from the Mother plant.

Week Five: Collect Phenotype Data and Purify DNA

- Examine the color of the hypocotyl of the plants and record each plant's phenotype (purple or nonpurple).
- Purify DNA from the seedling tissue following the protocol in your lab manual.
- Start PCR.

Week Six: Gels and Data Analysis

- Run gels with your PCR products.
- Use the bands on the gel to determine the genotypes of your plants for the DNA marker.
- Use the hypocotyl color data to deduce the genotypes of your plants for anthocyaninless.
- Determine if you can exclude either Alleged Father and present your group's results to the class.

Questions to Guide Data Analysis

The following series of questions guide the student in analysis of the data:

Marker *D9BrapaS4*: Symbols for alleles _____

Genotypes: Use your data to determine the genotype of each individual

Mother _____

Child _____

Alleged Father #1 _____

Alleged Father #2 _____

Of the Child's alleles, which **could** have come from the Mother? _____

Of the Child's alleles, which **must** have come from the Mother, and why?

Of the Child's alleles, which may have come from its father, whoever that is? _____

Based on the above, can you exclude Alleged Father #1? Why or why not?

Based on the above, can you exclude Alleged Father #2? Why or why not?

Marker *anthocyaninless*: Symbols for alleles _____

Genotypes: Use your data to determine the genotype of each individual

Mother _____

Child _____

Alleged Father #1 _____

Alleged Father #2 _____

Of the Child's alleles, which **could** have come from the Mother? _____

Of the Child's alleles, which **must** have come from the Mother, and why?

Of the Child's alleles, which may have come from its father, whoever that is? _____

Based on the above, can you exclude Alleged Father #1? Why or why not?

Based on the above, can you exclude Alleged Father #2? Why or why not?

Comparing the two markers. Were there any differences in the conclusions that you reached with the two markers tested?

Materials

The following is a list of the materials needed. More details can be found in the instructor's guide at https://files.oakland.edu/users/wendell/web/Teaching_resources.html.

Advance preparation 17 days before the mating: sow seeds of parent plants

Planting and growing:

- In addition to the instructor's guide, directions for planting and growing can be found at the following page in the Wisconsin Fast Plants web site http://www.fastplants.org/how_to_grow/planting_fertilization.php.
- To live up to their Fast Plant name, the plants require constant and intense illumination from fluorescent lights. Light bank systems can be purchased from Carolina Biological Supply or you can build your own using the instructions here http://www.fastplants.org/pdf/grow/lightbank_construction.pdf.

Materials for planting:

- Seeds of DWRCBr91, DWRCBr53, and F1 (hybrid of 76 and 53)
- Seeds for phenotype reference plants
- Light bank with six 40-Watt lamps
- Water wick system
- Distilled water
- 2 x 2 inch peat pots (such as Jiffy Pots)
- Watering cans made by punching a small hole in the lid of a 16 ounce plastic soda or water bottle
- Potting soil (Use a light seed starting mix. We use Bordine's.)
- Osmocote fertilizer pellets
- Vermiculite
- Labeling stakes/tags
- Markers (fine point Sharpies)
- Bamboo skewers (small skewers you get in packs of 100 at the grocery store)
- For each group, you will need one Mother, one Alleged Father #1, and one Alleged Father #2 that are all in bloom on the first day of the lab. I always start at least 30% extra pots of each strain because not all plants develop at the same rate. Then, the day before lab, select the best plants for the class.

Materials for Week One

- For each student group:
 - One plant of Mother, Alleged Father #1, and Alleged Father #2 staked and flowering.
 - Cotton swabs (Q tips)
 - 1.5 mL microtubes (aka Eppendorf tubes), one per student
 - Fine point permanent markers (such as ultrafine point Sharpies)
 - Scissors
 - Forceps

- For each student:
 - Charts for students to record data
 - Insure that they follow the manual's instructions on assigning ID numbers
 - Access to online illustrations of plant anatomy and life cycle or handouts
 - Control plants labeled by their color phenotype
 - Freezer space to store the tissue samples
 - Storage box for microtubes

Between Weeks Four and Five

Three to four days before the week five class, set Child seeds on wet filter paper under bright light to sprout:

- Envelopes to store extra seeds
- Petri dishes
- Blotter paper (3 mm paper)
- Distilled water

Materials for Week Five

Supplies and equipment needed for DNA Purification:

- Micropipettors (P1000 and P200 and P10/20) for each group
- Microcentrifuge
- Water bath set at 65 °C and warmed up before the start of class
- Mortar and pestle for each student
- 1.5 mL microtubes; minimum of three per student
- Videos of how to grind tissue using mortar and pestle <http://youtu.be/RNFT-VM05V4>
- Storage (refrigerator) space for student samples
- Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA www.qiagen.com)
 - Buffer AP1
 - RNase A
 - Buffer AP2
 - Buffer AP3/E
 - Buffer AW
 - Buffer AE
 - DNeasy Mini Spin Column

Supplies and equipment needed for PCR:

- P10 pipettor
- Thermal cycler
- 2X Taq Premix (Syzygy Biotech, Grand Rapids, MI; www.syzygybiotech.com)
- PCR primers
- 200 µL PCR tubes (Get tubes of four different colors, or three colors plus natural.)
- Racks that can hold 200 uL PCR tubes
- MilliQ water

PCR Protocol: 94 °C for 2 minutes, followed by 25 cycles of 94 °C for 30 seconds, 61 °C for 60 seconds, 72 °C for 60 seconds, finally after the cycles a polishing step of 72 °C for 4 minutes

Materials for Week Six

Equipment and supplies needed for gels:

- apparatus for agarose slab gels; “minigel” system
- combs: 1.0 mm thick with 10 or 12 wells
- power supply
- 10,000x SYBR Safe DNA Gel Stain (Molecular Probes, available through several vendors.)
- agarose gels: 1.2% agarose in TBE with SYBR Safe added when casting; use a 1 mm comb for best results
- DNA ladder
- sheets for the student to record what they loaded in each lane
- spare agarose gels and 1x sample buffer (dummy samples) for loading practice
- transilluminator: either uv transilluminator with shielding, or blue light transilluminator
- digital camera, or have students take photos with their phones and tablets

Notes for the Instructor

Information Available

A complete lab manual and in instructors supplement are available for download as a Word document at https://files.oakland.edu/users/wendell/web/Teaching_resources.html. We have also made instructional videos on key techniques and these are posted on YouTube® at <http://www.youtube.com/user/HumanGeneticsMustard>.

Choice of DNA Markers

If you use only one DNA marker, use *D9BrapaS4* because it has the largest size difference between alleles and is thus most readily resolved on a small agarose gel. If you want to use two DNA markers, we recommend *D9BrapaS4* and *D1BrapaS1* because they are on different chromosomes and will segregate independently. (They cannot be multiplexed, though)

Marker-Defined Strains

In order for this lab to work, there must be marker polymorphism between the Alleged Fathers. Although the DNA markers that we developed are polymorphic within the Fast Plants strains available from Carolina Biological Supply, in all of the Fast Plants strains we have surveyed one allele is present at a very high frequency and the other is rare in the population (Slankster et al., 2012). This makes it difficult to be assured of polymorphism. Therefore, we have bred marker-defined strains that are homozygous for different alleles of four DNA markers and for two Mendelian trait markers (Table 2). Seeds of these strains are available from Doug Wendell (wendell@oakland.edu). If you use strains DWRCBr53 and DWRCBr76 for the two Alleged Fathers, all cases will be informative, i.e. the students will always be able to exclude one of the fathers. We run the lab with one Alleged Father

being heterozygous to provide a greater range of possible results. To have one father that is heterozygous, we cross DWRCBr53 and DWRCBr76 and use the hybrid as one of the Alleged Fathers and DWRCBr53 as the other.

Table 2. Marker-defined strains.

	DWRCBr53	DWRCBr76	DWRCBr91
<i>D9BrapaS1</i>	2 / 2	1 / 1	2 / 2
<i>D9BrapaS4</i>	2 / 2	1 / 1	3 / 3
<i>D1BrapaS1</i>	1 / 1	2 / 2	1 / 1
<i>P a r k 9 - HaeIII</i>	cut / cut	uncut / uncut	cut / cut
<i>a n t h o c y - aninless</i>	<i>anl / anl</i>	<i>ANL / ANL</i>	<i>anl / anl</i>
<i>yellow-green</i>	<i>YGR / YGR</i>	<i>YGR / YGR</i>	<i>ygr / ygr</i>

Schedule Variations

The schedule we present for the lab is not the only possibility. The only non-negotiable factor is the time needed for the Mother to grow to flowering (17 days) and the time for seed development (3 – 4 weeks). We present the students with the flowering parents, rather than having the students grow them, in order to make the project fit in as one of several projects in our introductory biology lab.

Two major options are available for growing the Child. One option is to sprout the Child seeds and then collect data and tissue 3 days later. This is based on the method developed by the Wisconsin Fast Plants Program (<http://www.fastplants.org/pdf/activities/WFPgenetics-06web.pdf>) and has the advantage of not needing additional soil and pots and that the purple hypocotyl color phenotype is more strongly expressed in sprouts than in seedlings grown in soil. However, you can also grow the Child plant and collect phenotype data and tissue one or two weeks later. This has the advantage of giving the students more hands-on experience and also provides a Child that is grown under the same conditions as the parents.

If growing large numbers of parent plants is a problem, rather than having one set of parents for each group in your class, consider having one set of parents and growing many children. If one of the Possible Fathers is heterozygous and you genotype for multiple markers, a large number of Child genotypes are possible from one mating.

Gel Staining Methods

We have tried a variety of alternatives to ethidium bromide for detecting these markers, and these are summarized in Table 3. Images of gels stained with SYBR Safe or Gel Green can be easily captured with a camera phone or tablet computer (Fig. 4)

Notes from the Workshop at ABLE 2012

At the major workshop at the ABLE 2012 Conference, we discussed alternative methods and ways to deal with problems in managing the class. Below is a summary of contributions by attendees.

One attendee suggested using PlantSaver FTA cards for DNA isolation (Whatman, available through several vendors). Another suggested Instagene Matrix (Bio-Rad). We have not tried either of these yet, but it is worth looking into. If you try a different method, we suggest testing them on a small scale ahead of your semester.

Tube labeling can be a problem. This is especially true for the PCR reactions where there is very little area to label on a 200 μ l PCR tube. One attendee suggested using different color tubes to distinguish the different members of the dispute.

We discussed ways to manage setting up PCR reactions by our students particularly in large lab sections. It has been our experience that when a student fails to obtain genotype data, it is usually failure of the PCR. (If we take their DNA sample back to the lab and do PCR it almost always works.) There was general agreement among attendees on the need to have the students practice using the micropipettors before doing PCR. At least one attendee said that they use a “PCR assembly line” where students move through a series of supervised stations to obtain their reagents.

Possible Molecular Extensions

The combination of the DNA markers we have developed and the release of the *Brassica rapa* genome sequence provide the possibility of molecular extensions. There is now a finished sequence of *Brassica rapa* (Wang et al., 2011) and the *Brassica* database BRAD (Cheng et al., 2011) provides tools such as BLAST search and a genome browser.

Our DNA markers provide a bridge to the genome sequence. We have sequenced each allele of our markers and files of sequence data of these markers can be downloaded

from the same web site as we referenced above for the manual.

Table 3. DNA gel stains we have tried.

Stain	Manufacturer	Evaluation
SYBR Safe	Molecular Probes	Great. Bright sharp bands. View on UV or blue light transilluminator.
Gel Green	Biotium	Great. May produce a little bit of band distortion but not enough to interfere with genotyping View on uv or blue light transilluminator.
Ethidium Bromide	many	Mutagenic. Not as good for direct viewing in the classroom.
goGreen	Feldan	Not recommended because it affects band mobility proportional to intensity.
Quickview (visible)	Ward's Natural Science	Works well when PCR is working optimally. About half the sensitivity of fluorescent stains.
CarolinaB-LU (visible)	Carolina Biological Supply	Same results as Ward's.
Fast Blast (visible)	Bio-Rad	Sensitivity comparable to Quickview and CarolinaBLU but the bands fade with time.

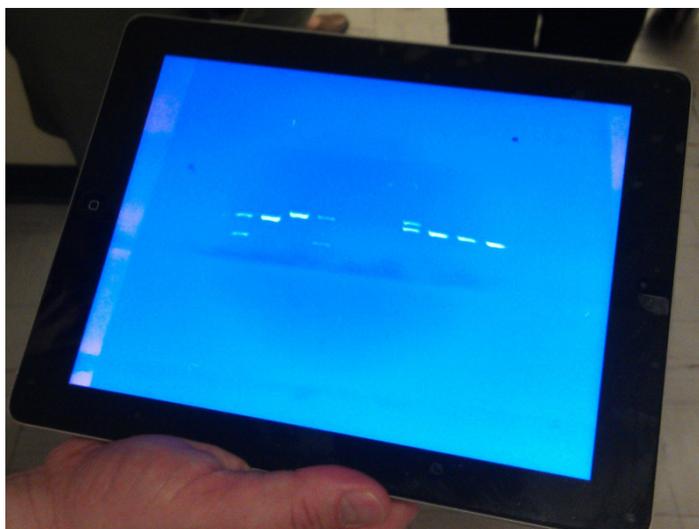


Figure 4. A workshop attendee shows the picture he took of a gel run in the workshop and stained with SYBR safe. Picture was taken using a tablet computer of the gel as is on the ultraviolet transilluminator.

Acknowledgments

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Doug Wendell earned his Ph.D. in Genetics at the University of California – Davis. He is currently an Associate Professor of Biology at Oakland University. His area of emphasis is genetics and he has done research on genes involved in tumor growth control and on developing materials for genetics laboratory classes. He has been teaching genetics laboratory and working with RCB_r and DNA markers for over ten years and collaborates with Dawn Pickard on science education projects.

Dawn Pickard earning her Ph.D. from Purdue University and then took a position as a zoology instructor at Anderson University, Anderson Indiana. In 1989, she moved to Oakland University, Rochester MI, where she currently is science education faculty. For the past several years, she has collaborated with Doug Wendell in developing genetics laboratory experiences for undergraduates.

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