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Human Genetics with Mustard: Use of DNA Markers for Paternity Testing in Rapid-Cycling *Brassica rapa* (Fast Plants Type)

Douglas L. Wendell[1], Dawn M. Pickard[2], Eryn E. Slankster[1], and Mary R. Belz[2]

Oakland University, [1]Department of Biological Sciences, [2]Department of Teacher Development & Educational Studies, 2200 N Squirrel Rd, Rochester MI 48309 USA
(wendell@oakland.edu; pickard@oakland.edu)

Students create a paternity dispute consisting of a child with two alleged fathers using rapid cycling *Brassica rapa*. The students purify DNA from the subjects and use PCR and gel electrophoresis to determine genotypes for variable number tandem repeat (VNTR) markers. Because of heterozygosity in the plants used, a variety of child genotypes are possible. In some cases, a clear conclusion of paternity exclusion can be reached but in others, neither father can be excluded, so students must learn to trace the inheritance of alleles from parents to offspring and evaluate the informativeness of their data. (**http://humangeneticsmustard.blogspot.com/**)

Keywords: Fast Plants, genetic markers, DNA fingerprinting

Introduction

Overview of the Lab

In this lab students use DNA fingerprinting-type genetic markers to attempt to resolve a paternity dispute consisting of a child with two alleged fathers. The students create this paternity dispute with rapid-cycling *Brassica rapa* (RCBr), also known as Fast Plants (Williams, 1997) by using a cotton swab to collect pollen from two plants (Alleged Father #1 and #2) and then using this swab to pollinate a third plant (Fig. 1). Seeds from the third plant (the Mother) are sown to produce a Child. Because RCBr are self-incompatible, i.e. they reject their own pollen (Musgrave, 2000), the child must be the progeny of the Mother and one of the Alleged Fathers.

At the same time that the students pollinate the parents, they collect and save a small amount of leaf tissue from each plant for later DNA analysis. Once the Child has grown sufficiently to produce leaf tissue (10 – 14 days), they purify DNA from all four individuals. They then perform PCR on the subjects with primers for one or more polymorphic DNA markers, and determine their genotypes by gel electrophoresis. Finally, they analyze the marker genotype data to determine which, if any, of the alleged fathers can be excluded.

DNA Markers for RCBr in a Teaching Lab

The markers that we use in this lab are based on variability in a segment of repetitive DNA. Such markers have been described under various names over the years including microsatellites, short tandem repeats (STR), and variable number tandem repeats (VNTR). The various terms correspond with repeat elements of different length: two or three bases in microsatellites, four to six for most STR, or dozens of bases in VNTR. However, they are all based on the same concept, namely, the marker contains a segment of DNA that is tandemly repeated and within a population there is variation in the number of repeats. The repetitive DNA is flanked by single-copy sequences that tag the marker to a specific chromosomal location (genetic locus). The variation in the number of repeats produces multiple alleles of that locus. The alleles are detected by PCR using primers anchored in the single copy sequence DNA flanking the repetitive DNA to produce fragments of variable length which are resolved by gel electrophoresis (Jeffreys *et al*., 1985; Jeffreys *et al*., 1988; Litt and Luty, 1989; Weber and May, 1989; Weber and Wong, 1993).

We developed our DNA markers for RCBr with qualities to make them suited to teaching lab use. STR are widely used in genetics for DNA fingerprinting and paternity testing, but with repeat elements of 4 to 6 base pairs (bp) (Butler and Reeder, 2011), the difference in the allele fragment sizes is typically small enough that they require more sophisticated methods such as polyacrylamide gels and they often require high sensitivity methods for detection. This is not a problem in research or forensics labs, where equipment such as automated capillary electrophoresis apparatus is available, but it does create a barrier to use in the teaching lab (Wendell and Pickard, 2007). Therefore, we have designed DNA markers based on VNTR-size repeats so that

the size difference between alleles is large enough to resolve on simple agarose gels. We searched *Brassica rapa* genome sequence data for candidate repetitive DNA elements with a minimum repeat size of 20 base pairs and designed PCR primers to amplify such sequences (Slankster, 2011). We then tested potential markers and selected only those that met the following criteria: (1) they robustly amplify a product, (2) they are polymorphic in RCBr, and (3) the size difference between alleles is large enough so that they can be easily separated on conventional agarose gels. We have developed several markers that met these criteria. Of these, we have found three, *D1BrapaS1, D9BrapaS1*, and *D9BrapaS4* to have alleles that can be easily separated on a 1.2% agarose gel and are amplified by PCR with high reliability. The PCR products for these markers are in the range of 300 to 600 bp. For a particular marker, the size difference between alleles ranges from 50 to 150 bp.

Multiple Possible Outcomes and Questions of Informativeness

The paternity testing experiment can be simple or complex depending on the genotype of the plants chosen to be the Alleged Fathers. If each Alleged Father is homozygous for a different allele of a VNTR marker then one father can always be excluded with certainty (Fig. 2A). However, more complex and interesting cases can be created if one of the fathers is heterozygous and shares an allele with the other. Such a setup creates multiple possible outcomes with differing degrees of informativeness. Fig. 2 parts B and C show possible results when one of the alleged fathers is heterozygous. If multiple student groups in class perform the lab, sometimes the students will obtain a child that is homozygous for the upper allele allowing Alleged Father #1 to be excluded. However, most of the time, the Child will inherit the shared allele and the students' analyses must lead to the conclusion that neither father can be excluded. An even greater set of possibilities can be had if multiple markers are used.

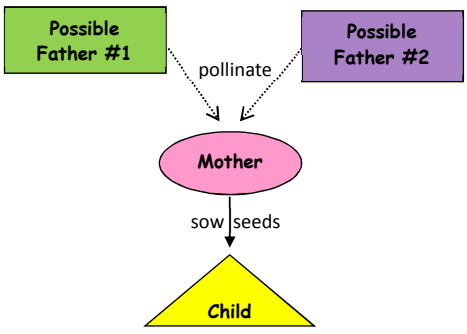

Figure 1. Creating a paternity dispute with RCBr.

The presence of cases shown in Figure 2B, C means that before they can perform paternity exclusion, the students must consider the informativeness of their data. A marker is only informative if the inheritance of a given allele can be traced from a specific parent to offspring. The students must realize that even though all of their PCR reactions and gels work perfectly, they still may not be able to make a conclusion about paternity.

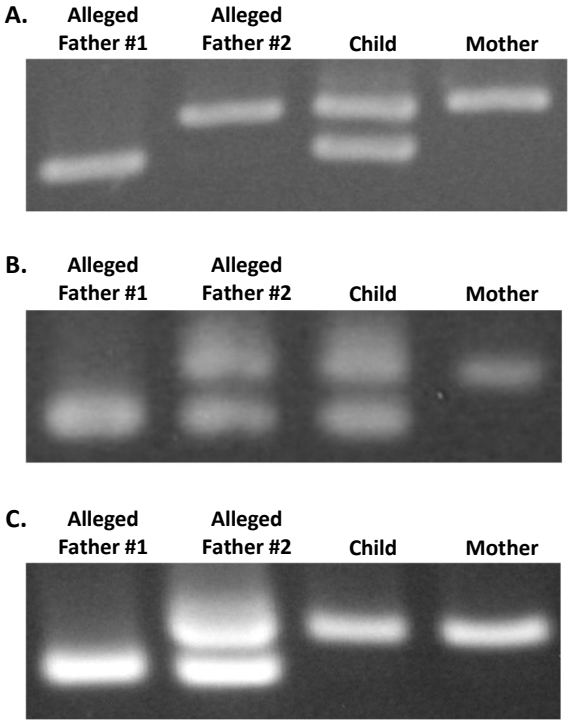

Figure 2. VNTR marker *D9BrapaS1* PCR products from paternity dispute cases run on a 1.2% agarose gel at 150 V for 40 minutes. **A**. A case where a clear paternity exclusion can be made because the Alleged Fathers are homozygous for different alleles of the marker. The Child is heterozygous and must have inherited the upper band allele from the Mother, and therefore the lower band must have come from the Child's father, whoever that is. Because Alleged Father #2 does not have that allele, he can be excluded. **B.** A case where one father is heterozygous and the two fathers share an allele. The Child has the lower band allele which could not have come from the Mother, so it must have come from its true father, whoever that is. Since both Alleged Fathers have this allele, neither can be excluded. **C**. A case with the same alleged father genotypes as in part **B** but this time the child inherits the allele unique to Alleged Father #2, so Alleged Father #1 can be excluded.

Student Outline

Learning Objectives, Conceptual

The completion of this lab's objectives will require the student to use important reasoning skills.

- How to translate bands on a gel into a genotype.
- The use of deductive reasoning to determine
  - which allele(s) the child could have inherited from its mother
  - which allele(s) the child could have inherited from its father
  - which father (if any) can be excluded

Learning Objectives, Technical

In this lab, students learn the following fundamental techniques of genetics and molecular biology:

- DNA purification
- PCR
- Gel electrophoresis

Lab Timeline

Below is the progression of experiments, based on a lab class that meets once per week.

2 ½ weeks prior	Instructor sows seeds
Week 1	Create the paternity dispute by pollinating the Mother plant with a mixture of pollen from two Alleged Fathers. Save tissue from the parents.
Week 4	Collect seeds from the Mother and plant the seeds that will be the Child.
Week 6	Collect tissue from the Child. Purify DNA of all subjects
Week 7	Quantify the DNA and start PCR for markers.
Week 8	Run gels and determine genotypes. Perform paternity exclusion.

Questions to Guide Data Analysis

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

Marker: D1BrapaS1 Symbols for alleles: _____

Genotypes: 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?

Notes for the Instructor

Materials and Information Available

In addition to developing markers, we have written and tested protocols to perform this lab, which we make freely available. Information on the VNTR markers including primer sequences and protocols for performing these experiments are available at **http://humangeneticsmustard.blogspot.com/**. We have also made instructional videos on key techniques and these are posted on YouTube at **http://www.youtube.com/user/HumanGeneticsMustard**. Although seeds for Fast Plants are available from Carolina Biological Supply, we have developed strains with defined genotypes to give the instructor control over the possible range of results. These seeds are available upon request from Doug Wendell (**wendell@oakland.edu**).

Procedures and Alternative Procedures

The protocols available at the above web site are the result of ten years of classroom use to find methods that are reliable in the hands of novice users with very basic equipment (Wendell and Pickard, 2007). The paternity testing project was originally used in a sophomore/junior level Genetics Laboratory course but is now being used in the freshman Biology Laboratory.

To purify DNA we use a protocol involving alkaline lysis, chloroform extraction, and ethanol precipitation. The use of chloroform may be a problem for some teaching lab situations. We find that spin column kits also work well, though they are more expensive. We have used the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and the Cartagen Rapid Homogenization Kit (Cartagen, Seattle, WA) with success.

To quantify the students' DNA preps, we have developed a method where 1µL is spotted on to a membrane and then stained with Fast Blast DNA Stain (BioRad, Hercules, CA). The intensity of the blue spot is compared to standards for an approximation of DNA concentration that is adequate for setting up PCR and avoids the need to have a spectrophotomer or fluorimeter available to the lab.

When we use this lab in our own classes, we stain the gels with ethidium bromide. However, we can also detect the bands with visible stains, though we find the sensitivity to be about one-half that of ethidium bromide (Fig. 3).

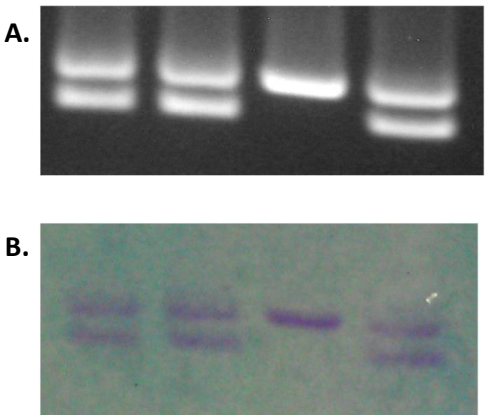

Figure 3. It is possible to detect these markers with less hazardous methods. **A.** A gel stained with ethidium bromide. **B.** The same gel stained with Fast Blast DNA Stain (BioRad, Hercules, CA).

General Tips

If the instructor plants the seeds for the parents in advance, so that they are in bloom when the students encounter them, the plant growth portion of the project can be done in five to six weeks. After that, an additional two lab sessions are needed to purify DNA, perform PCR, and run gels.

Labeling of samples and accurate record keeping is especially important because each plant in the paternity case is a genetically distinct individual. We and other users of this lab have experienced cases where students do not clearly label samples or use a labeling that they later cannot interpret. Therefore we have taken to dictating a simple procedure using **M** for mother, **P1** for alleged father #1, **P2** for alleged father #2, and **C** for the child. If there are multiple groups in a lab section, we use a Roman numeral to distinguish each lab section.

The brand of Taq polymerase is important. Of various brands we have tested, we have obtained good results with Invitrogen Accuprime Taq (Invitrogen Corp., Carlsbad, CA), illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences Piscataway, NJ), and Syzygy Biotech Taq Master Mix (Syzygy Biotech, Grand Rapids, MI). We acknowledge that other brands available that we have not yet tried may also work.

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About the Authors

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 RCBr 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.

Eryn Slankster earned her B.S. and M.S. in Biology from Oakland University. Her master's thesis research was the development of DNA markers for RCBr. She is currently a doctoral student in Biomedical Sciences doing research on cancer stem cells. She has also been a graduate teaching assistant in genetics laboratory.

Mary Belz earned her B.S. in Elementary Education and her M.Ed. in Educational Studies from Oakland University. She is currently a post graduate student at Oakland University where her current research focuses on curriculum for exceptional students in general education. She has taught Instructional Design and Assessment at OU. She is also the producer of Podcasts for Leaderful Schools.

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