Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Vol. 35, 281-289, 2014

Detecting Genetic Polymorphisms in Different Populations of Bean Beetle (*Callosobruchus maculatus*)

Jennifer McMahon Smith and Karen A. Hicks

Kenyon College, Department of Biology, 202 N. College Rd., Gambier OH 43022 USA
(**smithje@kenyon.edu; hicksk@kenyon.edu**)

Differences at the DNA sequence level are intrinsically important in categorizing groups of organisms. This exercise introduces students to DNA extraction and PCR, with which they will compare different populations of bean beetles (*Callosobruchus maculatus*). Using primer sets generated from the *Callosobruchus* genome, students compare DNA amplification patterns via electrophoresis. DNA regions selected for amplification have been identified as containing microsatellites, which often contain numerous polymorphisms useful in assessing genetic similarities. Students are asked to consider how separation of populations may affect changes at the DNA level. This exercise offers participation in new research, and contributes to bean beetle biology.

Keywords: Bean beetle, *Callosobruchus maculatus*, microsatellites, STR, PCR

Introduction

This activity was prepared for an Introductory Biology lab.

Pre-laboratory Preparation

The instructor will need several separate populations of bean beetles, all of which have emerged from their seeds at the same time. Because bean beetles raised on different beans have varying developmental periods, these cultures will need to be started several months in advance in order to have cultures that are ready for this lab. We have also had limited success in extracting DNA from dead beetles.

By the time students have reached this exercise, most have been introduced to DNA structure and replication in a Biology lecture class, and they should have learned how PCR works prior to this exercise. Our students will have taken an on-line quiz following a tutorial on how PCR works. It is important that they understand how we are able to target particular regions of DNA, amplify them, and are able to compare them to other DNA samples via electrophoresis.

Our students at Kenyon will already be familiar with the life history of the bean beetle, as they have been introduced to the system during an earlier laboratory exercise, but for classes who have not used bean beetles before, a brief introduction to the life history of bean beetles is advised. For further information about bean beetles, please visit **www.beanbeetles.org**.

Introducing Students to the Lab

To begin the exercise, the instructor might ask students to come up with ideas for how we as biologists classify groups of organisms. Students may respond by proposing means of classification such as the region where they are found, coloring, diet, genetics, etc. Most of the ways to sort organisms that students will most likely be based upon phenotypic traits. Ask your students to observe the different bean beetle populations at their tables - can they see any differences between these groups? Students may offer observations on variation in color or behavior between these groups, but any differences will be subtle at best. This is a good time in which to discuss how to qualify differences that we can't necessarily see, that are present at the molecular level. Some molecular differences, of course, can be seen- through different phenotypes for a particular trait. But the genome in most eukaryotes contains large regions of non-coding DNA. This DNA too can contain variation particularly in the length of these non-coding regions; the term *polymorphism* describes the variability of DNA at a particular locus.

While there are many classes of DNA polymorphisms, the types that our students will be analyzing are Short Tandem Repeats (also called STRs or microsatellites). These are relatively small repeat units of 2-6 nucleotides in length. These repeats can be formed by mistakes made by the DNA Polymerase during DNA replication, and can be passed down to offspring from parents.

These changes in STR length can be visualized using PCR. Using the same DNA primers pairs on DNA samples from individuals from different populations, we can look for alterations in the size of the PCR products that are generated.

These size differences can be seen based upon how quickly the DNA bands run through an agarose gel; larger products run more slowly than smaller products. Students may be asked to evaluate whether this method of analysis will definitively determine whether one group of individuals is different at the genetic level from another group.

In addition to the DNA samples that will be used during PCR, students should consider what control should be used in this experiment. This may take some time, but they should realize that PCR should only show products when there is template DNA added to the PCR reagents. Contamination and primers binding to each other can also show up in a DNA gel, which is why control samples that contain everything except the DNA should also be run. The details of DNA extraction and PCR are outlined for the students. Remind students that they must follow directions closely, pipette carefully, and wear gloves.

Our students initially used 4 populations of bean beetles-LB, FM strains, as well as LB progeny that were raised on either adzuki (Ad) or black-eyed peas (BEP) for over 20 generations. More recently, we have obtained cultures from South India and Burkina Faso, which have replaced the Ad and BEP strains in the lab exercise. Students work as pairs to select a primer pair from Table 2 that they will use in their PCR reactions. The instructor will need to know which primer has the lowest melting temperature (T_m) of all those that are used by the class in order to program the correct annealing temperature on the thermocycler. The annealing temperature used to amplify the class DNA should be set 5°C lower than the lowest T_m. Better results can be attained when primer sets chosen by the class have similar T_m's (avoid working with a wide range of T_m).

Experimental Design

Because changes to STRs are randomly occurring events, and since all bean beetle populations have a common origin, it is possible that differences in STR regions will exist between different bean beetle populations. Moreover, it is expected that these polymorphisms will be more prevalent in populations that have been separated from each other (geographically/chronologically) for greater periods of time than in populations separated more recently.

Students should be able to:

- Predict outcomes for this experiment - which populations are more likely to be genetically similar? Which are expected to be less genetically similar?
- How do we determine greater or lesser genetic similarity based upon our results?
- What sort of control should we use in this experimental setup and why do we have a control of this sort?

Sample Analysis

The students' results will be determined the following week, when they will run their samples on 3% agarose gels, and then visualize the DNA bands under UV light. Using DNA molecular weight standards, they can calculate the size or sizes of DNA bands present for their samples. They should also look at the results of other groups in order to see how many of the primer sets used showed bands of different sizes for their different bean beetle populations. Using PCR, we are able to only detect differences in sequence length, rather than nucleotide substitutions. This exercise can lead to further discussions of whether all changes at the DNA level would be able to be determined using our methodology, and if not, what additional methods should be employed.

If DNA amplification is successful, there are two possible outcomes: either a locus is polymorphic, or it is not. If a locus is polymorphic, it may be so because of heterozygosity in an individual beetle. A locus from an individual beetle is polymorphic, i.e. a heterozygote, if two alleles (bands) are visible in a single lane. Allelic variability between beetles from separate populations is also a possible outcome. Polymorphisms between beetles from different populations are identified as amplification products with different sizes in different lanes.

We have found that artifacts from PCR error are not unusual in this laboratory, and the instructor should try to distinguish these from bands that should be analyzed in order to steer students away from trying to analyze the wrong products. For example, "stutter" products may show up as a function of Taq Polymerase error. Stuttering appears as bands that are slightly smaller and less intense than the principal band(s). Additionally, smears that extend down the gel lane are also common. These are likely due to the quality of the DNA prep, and may be caused by contamination of the DNA sample by non-soluble parts of the extraction, e.g. insect body parts. Lastly, depending upon primer construction, primer-dimer formation may be visible as low molecular weight bands. Raising the annealing temperature during amplification and encouraging the use of primer sets with similar melting temperatures may eliminate these additional bands.

Student Outline

Although insects comprise as much as 20% of the species on Earth, comparatively little has been done to characterize most insects at the genetic level. At present, only about 40 insect species' genomes have been completely sequenced, and of these only one is from the Order *Coleoptera* (beetles). In September 2011, a new genome sequence for the bean beetle, *Callosobruchus maculatus*, was reported, and the features of this genome are only now starting to be studied.

Differences at the DNA sequence level are intrinsically important in categorizing one group of organisms relative to another. Variation in nucleotide sequences and gene organization can be used to distinguish sub-populations of the same species (intraspecific genetic variation). Scientists are often interested in understanding what types of variations exist in the DNA code between different populations in order to establish relatedness, rates of migration, etc. (Selkoe and Toonen, 2006) In this exercise, we will compare genetic variation between different populations of the bean beetle. As you have learned earlier in this course, this insect is native to both Asia and Africa, and has spread throughout the tropics. We have four different populations or strains that we will be comparing: "LB" strain, "FM" strain from S. India, and two populations of the LB each of which have been reared on a different type of bean for over 20 generations.

How will we be able to determine the genetic variability between our populations? First, we must understand that there are many different types of DNA modifications that contribute to genetic variability. In this experiment, we are going to look for variability in regions of DNA that are called *Short Tandem Repeats* (STRs) or *microsatellites.* STRs are DNA patterns of repeating nucleotides. These repeat units can vary in length from 2 to 6 base pairs (e.g. $(GAT)_n$), and are generally in the non-coding regions of the genome. Each STR can vary in length at the same place in the genome (locus). This variation is due to changes in the number of repeats. STRs occur through naturally occurring "slippage" of the DNA replication machinery, and are heritable. The heritability of these errors made during DNA replication can help us identify relatedness of individuals and of samples from different populations. This is sometimes referred to as "DNA fingerprinting".

How can we determine relatedness using STRs? First, we must assume that these DNA repeating patterns can be inherited by offspring from their parents. We should also assume that individuals from different populations may have different STRs, depending upon how genetically divergent they are. Thirdly, we can detect differences in the STRs at different locations because of their differences in size. By amplifying the DNA using the polymerase chain reaction (PCR), we can amass enough DNA to ascertain whether there are differences in the length of the STR region. We look for changes in the length of DNA products by running the products out on agarose gels to look for differences in migration of the DNA products. Using microsatellites to determine genetic diversity is a very common procedure and has been used across a broad range of living systems, including insects (Fritz and Schabel, 2004; Lavendero and Dominguez, 2010).

Materials

During the first week of this experiment, we will isolate DNA from the different bean beetle populations, will choose the primer set with which you and your partner is going to work, and will set up the PCR reactions. You will have four populations of beetles that you will use in this experiment- LB (raised on mung bean); FM (raised on mung bean); Ad (LB strain raised on adzuki beans); and BEP (LB strain raised on black-eyed peas). The origins of the LB and FM strains are geographically distinct from each other, and the LB strains in particular have been isolated from each other for over 20 generations. Using the protocol described below, you will extract DNA from these four types of beetles, and prepare your PCR reactions.

Next week, we shall separate the DNA strands that were amplified via PCR on agarose gels so that we can measure and compare the size of the products.

Experimental Design

Work as pairs for this experiment. You and your partner should coordinate how you will proceed for this experiment.

Observe the four populations of beetles prior to extracting DNA. Do you see phenotypic differences between these populations? Would you expect to see phenotypic differences? Why or why not?

- Predict outcomes for this experiment - which populations are more likely to be genetically similar? Which are expected to be less genetically similar?
- How do we determine greater or lesser genetic similarity based upon our results?
- What sort of control should we use in this experimental setup and why do we have a control of this sort?

In September 2011, the genome for *C. maculatus* (LB strain) was first publicly reported. Using a search program, Phobos (**http://www.ruhr-uni-bochum.de/ecoevo/cm/cm_phobos.htm**), a list of genome DNA segments ("Contigs") that contained repeat sequences was created. Of these, 29 were selected, and primers were generated using a second program, Primer3 (**http://frodo.wi.mit.edu/primer3/input.htm**). You and your partner will select a primer set from Table 2 - what size product are you expecting to amplify? What does the repeat unit look like in the piece of DNA that you are studying?

Methods (Nishiyama, 2004)

- Each research group will use one bean beetle from each of the populations of beetles on the table. Place each beetle in a separate microcentrifuge tube with 30 µl TE buffer.
- Immediately break up the beetle body with a sterile toothpick, being certain that the body cavity is completely mashed up. Some cutin may stick to the toothpick.
- Freeze with liquid nitrogen, allow to thaw at room temperature, and repeat the freeze/thaw cycle once more.
- Heat samples at 68°C in the heat block for 10 minutes and centrifuge at 5000 rpm for 5 minutes at room temperature.
- To set up your PCR reactions, you need to make a Master Mix that contains everything except for the template DNA. To make a Master Mix sufficient for five reactions (four experimental samples and one control) you will need 54 µl Supermix (Invitrogen) and 3 µl Primer pair mix (10 µM stock).
- To set up each PCR reaction in the small 0.2 ml tubes, you will require 9 µl of the Master Mix and 1 µl of your DNA. What goes into the control reaction?
- The thermocycler in which the PCR will take place has been programmed for you - the cycling conditions are as follows:

1 min	94°C	
15 sec	94°C	} 40 cycles (temperature depends on primers used)
15 sec	## °C	
1 min	72°C	
Hold at	4°C	

Next week, we will run out your PCR samples on agarose gels. What would you expect to see on the gel if polymorphisms are present in the region that you amplified? Does the presence of STR polymorphisms in one region of the chromosome guarantee polymorphisms in other STR regions? Since we are comparing four populations, between which populations of bean beetles might there be fewer polymorphisms? Between which populations might you expect to see more polymorphisms?

Assignment

Following the results of next week's gels, you are to write a short paper (Results and Discussion) outlining the results of the laboratory class PCR experiment. This will require you to observe the results of other gels as well as the one onto which your samples were loaded. To help you organize the class results, use the table on the following page.

Some questions to consider when writing your paper:

1. What was the size(s) of the product(s) you were able to amplify with your primer pair?
2. Were you able to see any polymorphisms with the primer pairs used in your lab section? If so, what sorts of changes did you detect?
3. Does a band of similar size in each of your PCR reactions indicate DNA that is 100% identical? Why or why not?
4. What do your results suggest as far as how genetically similar these populations of bean beetles are?

Table 1. Summary of genetic variability of bean beetle cultures. Please use class data in order to complete this table.

Primer No.	Polymorphic? Y/N	# of alleles per locus	Size of fragment(s) LB FM Ad BEP
	% Polymorphic		

Table 2. Primer pairs surrounding selected STRs from *Callosobruchus maculatus* genome (female). Each group uses one primer pair.

Primer Pair	Contig #	Repeat Sequence Unit	Left Seq.	Right Seq.	Exp. Frag Size (bp)	Lowest T_m (°C)
1	31898	AG	GCAATGATGACTGCCTTTGA	TGCAAATTAACGTAAGTGAGAGTCA	186	58.4
2	34449	AG	TTCAGGTTTTCTTTATCCATTTGA	TTTCTCCATTTCTCCATTTCTACA	127	56
3	259859	AAG	TCTCCTTCCTCTAATACCTCTACTCC	TCATTGATGTGGGTAAGAATGAG	292	53.4
4	255505	AAAT	GTTGCTCAGCTCCAAGCTCT	TGCAATACGCAAATCATTTTAT	127	59.2
5	37894	AAG	CGAGCTCTCACAGGTAAATTTCT	AACCGCATAGGTGTCTCTGG	177	61
6	50628	AAAT	GGATTTGTTTCTGTTTACGGAGT	ATCCATGACGTCGGGATAAT	124	58.4

Primer Pair	Contig #	Repeat Sequence Unit	Left Seq.	Right Seq.	Exp. Frag Size (bp)	Lowest T_m (°C)
7	67295	AG	AGGTTG-CAGAAGGAG-GCTCT	CCGTCCTG-GAGCCATATCT	142	62.3
8	94421	AAGAGG	TGAATGGTGT-TAAAACTAAT-GAACAA	TCACAGAAT-TACAACTTTCA-CAGTA	264	56.7
9	94990	ACAGAG	CGCTGTTT-GAACGT-GAATTT	CCACACTCAGT-GAGAGAGAGA-GAG	199	56.3
10	99468	AAAT	TTATCAGT-GATCTGAAGA-CACCTC	AGTTCGACTAT-TATGAGTGC-GTTA	173	59.4
11	107719	ACAT	GAGCTTT-TATTCAACTTG-CATTG	TAGCAAGATTC-CACCCGAAC	111	57.4
12	120552	AAAT	TTCGTGATA-ATCCTATCTGT-GATTT	TGGTTGATC-GGTGTA-AAACTTG	300	58
13	121086	AAG	GTCCGGAGCA-GATAAAGCAA	GTGTAAAGAT-GCTCTTGCTA-ACG	150	60.4
14	121429	AAG	TGTTAAACTC-GCGTTTGTGG	CCGTCAAAACT-GCGTCATAG	127	58.4
15	125823	AATT	TTCTGTA-AGCATCCTA-AATGCAA	AGGTGAC-CAATTGTTTG-GAA	136	56.3
16	132879	ACAG	GCCTTTTTCT-GTTTACTAA-CAGTTCA	ATCGTTTGAT-GCCGGCATTA	209	58.4
17	141545	ACAT	GGTCTCACA-CATCCGCAATA	TGACCAA-CATAC-GATTTTCTTCAC	131	59.4
18	143022	ACAG	TCTACATAAC-GAAAATCCT-GTCG	GAAGAACGC-CAACAGACAGA	200	59.2
19	143214	AG	CTCTGTCTC-GATCTCTCTTT-GC	TCATTATT-GATTTGCATTA-CAGAAA	146	54.7
20	170737	ACAG	AACGGTTACT-GAGATTAC-GAAGC	ACGAAAATC-CACGTCACTCA	119	58.4
21	171469	ACTAGT	CCGTACGCTG-GAAGAAAAGA	TGGAGTTATC-GACCGTACCA	204	60.4
22	182441	AATC	TTTTGTAGTTT-GTTTTTATATCT-GCA	AGGTTGGT-CATGGGGTT-GAT	181	55.5
23	197614	AAG	GCTTGACGCG-TAGTTGTGAA	CAAAAGAAC-GACGTAAA-CACG	188	58.7

Primer Pair	Contig #	Repeat Sequence Unit	Left Seq.	Right Seq.	Exp. Frag Size (bp)	Lowest T_m (°C)
24	210923	AG	TGTCTGACGTTTGACGTTCC	AAATCGGGTGACTGAGTTT	136	56.7
25	227899	ACAG	CAGCGGTTACTGAGATTAGATGG	CGAAAATCCACATCACTCATTT	237	57.1
26	229686	AAGGTC	AATGTATGTTCTGGAGGAGCAA	GGTTTGATTCCTAGTTGAGGATTT	174	58.9
27	231676	ACAG	AGCGGTTGCTGAGATTACGA	CGAAAATCCACGTCACTCATT	122	58.7
28	237701	ACAG	AAAAATCCATGTCACTCATTTCC	GCCTGTTTATTATCTTCCGATAGC	155	57.4
29	251821	AAG	TTTTTCGACAAGATGTACACAAGG	GATACAGTATGGAAATTGAAAAGAGGA	150	59.4

Materials

For a Class of 30 Working in Pairs:

- Bean beetle cultures (8 each) obtained from Carolina Biological or private researchers
- Sterile toothpicks or sterilized plastic pestles - 4 per group
- Sterile 0.5 ml microcentrifuge tubes
- TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA)
- Liquid nitrogen
- Forceps
- Heat block (or can use PCR machine)
- Platinum Blue Supermix (Invitrogen Cat. No. 12580-015)
- Primer pair mixes (10 µM stock) (Eurofins/MWG Operon)
- Micro centrifuge
- Thermocycler (we used the PTC-100 from MJ Research Inc.)
- PCR reaction tubes, 0.2 ml volume (USA Scientific Cat No. 1402-2900)
- PCR Molecular Weight Marker (New England BioLabs Cat. No. N3234S)
- Agarose (3% gels). We used a 50/50 mix of agarose for our gels- Invitrogen UltraPureAgarose 1000 (Cat. No. 16550-100) and Sigma-Aldrich Agarose Type 1, Low EEO (Cat. No. A6013)
- 10 X TBE Buffer (890 mMTris, 890 mM Boric acid, 20 mM EDTA, pH 8.0)- used at 1X strength for DNA gel and running buffer
- Ethidium bromide (final concentration 5 µg/ml)
- Power supply
- Gel rigs for electrophoresis
- UV transilluminator
- Micropipettors (P200 and P20) and sterile pipette tips

Notes for the Instructor

A few aspects of laboratory performance are critical in order to achieve success with this protocol. First, if wooden toothpicks are used to grind up the beetle, extended use will cause the toothpicks to absorb the extraction buffer. Grinding the beetle should be done quickly to avoid this, or the use of plastic pestles can be employed to eliminate this issue. Secondly, accurate pipetting is essential to the success of this experiment and for using the volume of primers and Supermix recommended in the protocol.

Care should be taken when using ethidium bromide, and gloves should be used to handle equipment and gels that come into contact with this reagent. Proper disposal methods should be employed. Band detection has also been successful using SYBR Green (Life Technologies).

The primers selected for this study flank microsatellite regions of the bean beetle genome, and were designed without certainty that any locus thus identified was polymorphic. As a result, several primers that were originally selected do not appear to be polymorphic in the beetle populations used. Of those we have successfully amplified, primer pairs 5, 7, 13, 23, 25 and 27 seem to show polymorphisms within at least one population. We will continue to evaluate additional microsatellite loci in upcoming iterations of this laboratory.

Phobos, the program used to identify DNA segments with microsatellites, has instructions and support at its website (**http://www.ruhr-uni-bochum.de/ecoevo/cm/cm_phobos.htm**). For this lab, default parameters were used except as follows: extend exact search; maximum score reduction: 30; repeat unit length: 2-6 bp; minimum length of satellite: 0 + 2, not less than 30; minimum score: 0 + 0, not less than 0. Repeat regions 50-200 bp in length were selected from those reported by Phobos, and primers were generated using Primer3 (**http://frodo.wi.mit.edu/primer3/input.htm**). Note that the specific Phobos parameters used may not be ideal for other genomes or purposes.

Acknowledgements

The authors would like to thank Dr. Larry Blumer and Dr. Christopher Beck for their continued support of bean beetle research. The authors would also like to acknowledge the Introductory Biology students at Kenyon College whose efforts helped to develop this laboratory, particularly Daniel Lipsey. This work was supported through the NSF (grants DUE-0535903, DUE-0815135, and DUE-0814373 to Morehouse College and Emory University, Atlanta, Georgia, USA).

Literature Cited

Fritz, A. H., and N. Schable. 2004. Microsatellite loci from the Caribbean Fruit Fly, *Anastrepha suspensa* (Diptera: Tephritidae). *Molecular Ecology Notes,* 4: 443-445.

Lavendero, B., and M. Dominguez. 2010. Isolation and characterization of nine microsatellite loci from *Aphelinus mali* (Hymenoptera: Aphelinidae), a parasitoid of *Eriosoman lanigerum* (Hemiptera: Aphididae). *Insect Science*, 17: 549-552.

Nishiyama, T. [Internet] 2004 July 15. Simple DNA extraction for PCR screening. Available from **http://www.nibb.ac.jp/~tomoaki/protocols/simplePCR**

Selkoe, K.A., and R. J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters*, 9: 615-629.

About the Authors

Jennifer Smith is the Lead Instructor and Director of the Introductory Labs in Biology at Kenyon College. Smith received her Ph.D. from The Ohio State University. Karen Hicks is an Associate Professor of Biology at Kenyon College where she teaches Genetics and Developmental Biology. Hicks received her Ph.D. from Massachusetts Institute of Technology. Smith and Hicks attended a Bean Beetle Curriculum Development Workshop in Atlanta, GA in 2011 through which this laboratory was developed.

Mission, Review Process & Disclaimer

The Association for Biology Laboratory Education (ABLE) was founded in 1979 to promote information exchange among university and college educators actively concerned with teaching biology in a laboratory setting. The focus of ABLE is to improve the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises. For more information about ABLE, please visit **http://www.ableweb.org/.**

Papers published in *Tested Studies for Laboratory Teaching: Peer-Reviewed Proceedings of the Conference of the Association for Biology Laboratory Education* are evaluated and selected by a committee prior to presentation at the conference, peer-reviewed by participants at the conference, and edited by members of the ABLE Editorial Board.

Citing This Article

Smith, J.M. and K.A. Hicks 2014. Detecting Genetic Polymorphisms in Different Populations of Bean Beetle (*Callosobruchus maculatus*). Pages 281-289 in *Tested Studies for Laboratory Teaching,* Volume 35 (K. McMahon, Editor). Proceedings of the 35th Conference of the Association for Biology Laboratory Education (ABLE), 477 pages.
http://www.ableweb.org/volumes/vol-35/?art=17