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Examining Genetic Diversity in Disjunct Populations using Random DNA Markers

Robert Sheehy and Judy Guinan

Biology Department
Box 6931
Radford University
Radford, VA 24141
rsheehy@radford.edu
jaguinan@radford.edu

Abstract: Molecular biological techniques are used to study naturally occurring genetic variation, and have greatly facilitated the understanding of problems in many areas of biology. In this lab, we examine two populations of terrestrial isopods in the Family Armadillidiidae using **R**andom **A**mplification of **P**olymorphic **D**NA (RAPD Analysis). RAPD's are robust and generally species-independent. Using this technique, we are able to detect and analyze genetic variation within and between two different populations of isopods. This lab exercise could easily be modified for use in an introductory course for majors or non-majors and would be suitable for any organism with low vagility.

Keywords: DNA, RAPD, genetic diversity, PCR, electrophoresis, isopods

Contents:

Introduction

Molecular biological techniques have become very useful in the study of plant and animal ecology, evolution, and conservation biology. These techniques are used to study naturally occurring genetic variation, and have greatly facilitated the understanding of many problems in behavioral ecology, population biology, and evolutionary biology. This lab serves as a capstone lab experience uniting principles students have learned throughout the semester. Content includes ecology, population biology, genetics, and molecular biology. Skills include microscopy, electrophoresis, molecular techniques (pipetting, PCR, solutions), graphing, and logarithms. We will examine two populations of terrestrial isopods in the Family Armadillidiidae ('Pill bugs' or 'Potato bugs' or 'Wood Lice') using **R**andom **A**mplification of **P**olymorphic **D**NA, (RAPD Analysis). RAPD's are robust and generally species independent. This lab can be modified and would be suitable for any organism with low vagility. Using this technique we are able to detect and analyze genetic variation within and between two different populations of isopods. This lab exercise could easily be modified for use in an introductory course for majors or non-majors.

Student Time Table

Day #1

Collect specimens from the field

Day #2

Pluck legs from isopods (15 minutes)
Add Legs to Extraction Buffer (10 Minutes)
Boil for 10 minutes (15 minutes)
Add DNA solution to PCR reaction (10 minutes)
Allow PCR to cycle (~ 3 hours)

Day #3

Mix PCR sample with loading dye (10 minutes)
Load gel (20 minutes)
Electrophoresis (~24 hours)

Day #4

Staining Gel (30 minutes)
Photograph gel (10 minutes)
Analyze results (50 – 90 minutes)

Materials

Materials needed for a class of 24 students working in groups of 4:

Equipment

- Thermo cycler (a programmable heating block)
- Micropipetter (1 – 5 μL and 25 μL)
- Microcentrifuge (optional)
- Power supply for electrophoresis (1)
- Electrophoresis chamber – (1, 20 cm gel capable of holding ~24 samples)
- Peristaltic or low volume pump (if using TAE buffered gel) (1)
- UV light box for detection of ethidium bromide stained DNA (1)
- Polaroid camera or photo documentation system (1)
- Tweezers for leg plucking – 1 per group of 4 students
- Ice bucket - one per group of from four to six students

Consumable Supplies

Item	Company	Cat #	Cost
Acetic Acid, Glacial, (500 mL)	Fisher	BP1185-500	$23.52
Agarose, high gelling temperature (100 grams)	Fisher	BP1356-100	$154.209
Bovine Serum Albumin (5 grams)	Sigma	B4287-5G	$39.30
Chelex 100 Sodium form (100 grams)	Sigma	C7901-100	$135.00
Cresol Red (5 grams)	Sigma	114472	$11.00
dNTP	Fisher	E0032003206	$115.00
EDTA, 1 kg	Fisher	AC118430010	$33.50
EtBr, 1 gram	Sigma	E7637-1G	22.20
Gloves, powder free latex	Fisher	11-462-68C	17.80
Lab Markers, 12 pack	Fisher	13-380-118	$16.64
$MgCl_2$ (1 kg)	Fisher	AC19753-0010	$38.30
Microfuge tubes 1.5 ml	Fisher	05-402-26	$30.00
Microfuge tubes thinwall PCR, 8 stirps	Fisher	21-236-68	$84.00
Mol, Wt. Markers, (e.g., 250 bp ladder)	Fisher	BP2552-100	$202.00
Pipet tips (blue) 1mL, 1000 count	Fisher	21-278-52	$16.77
Pipet tips (Yellow) 10-200 mL,1000 count	Fisher	21-278-51	$29.60
Pipet Tips 10 mL	Fisher	21-197-2F	$48.95
RAPD primers (Half kit (50 primers)	UBC		$132.00
Sucrose (1 kg)	Fisher	BP220-1	$44.63
Thermo stable DNA polymerase (e.g., Taq)	Fisher	FB-6000-10	$53.08
Tris Base (500grams)	Fisher	BP152-500	$38.46

- Indelible lab marker - one per group
- Microcentrifuge tubes (500 micro liter size) - one per student
- Agarose gel - number required varies, depending on number of lanes in gel
- Micropipette tips (barrier tips) - 3 per student
- Micropipette tips (normal) - 1 per student
- Gloves, disposable (reusable dishwashing gloves are ok) - one pair for anyone handling gel
- Ice water bath or crushed ice - one per group
- 5% chelex solution (500 mL per student)
- PCR master mix (20 mL per student. Should always make extra)
  - Nucleotides
  - PCR buffer
  - RAPD primers
  - Thermo stable DNA polymerase
  - Distilled H_20 (grocery store quality is acceptable)
- Molecular weight marker - 15 μL per gel (enough for three lanes)
- RAPD primers (available from University of British Columbia)

http://www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/

Notes for the Instructor

Overview

This laboratory exercise has been used to draw together various concepts that the students have been exposed to throughout the semester/year. This laboratory also allows us to integrate aspects of biology that students often feel are disparate and unrelated (e.g., ecology and molecular biology).

Day 1 (collection of Specimens)

Students may play a big role in determining which taxa are appropriate to use in this experiment. Perhaps there are species that have been used for laboratory analysis earlier in the course that may be appropriate and for which students already have some familiarity. The ideal taxa would have low vagility with relatively apparent natural barriers. Students should be encouraged to consider the natural history of an organism when choosing a taxon to examine. For example, our students have often wanted to use plant taxa because of their low mobility, often ignoring some aspects of their life cycle such as seed and/or pollen dispersal. Specimens can often be collected days or weeks ahead of time. Students may enjoy observing behavior of invertebrate taxa.

Day 2 (DNA extraction and PCR amplification)

- RAPD PCR primers are not species specific. Care must be taken not to contaminate the reaction with human cells. Gloves should be worn and at least one negative control should be included.
- Students should be reminded to keep all PCR reactions on ice prior to loading them in the thermal cycler.
- A thermal cycler with a heated lid is preferred as it avoids the necessity of an oil overlay. We have found that students have a difficult time loading samples when oil was used in the PCR.
- Samples may be frozen after the PCR reaction until it is convenient to proceed to the next step.

Day 3 (Electrophoresis)

- RAPD markers tend to be small and therefore require a gel of high agarose content (1.4 – 2.0%) and a long gel for adequate separation. Separation is also better if the gel is run at low voltage over a longer period. We generally run our gels at 20 – 25 volts overnight. Gels resolution may also be improved by using TAE electrophoresis buffer rather than TBE. The buffering capabilities of TAE are lower which requires that the gels be run with a circulator to move buffer from one side of the electrophoresis chamber to the other. TBE may be adequate in many situations.
- It is common to add ETBr to the gel and/or the buffer before electrophoresis. We have found however that resolution is improved if gels are stained after electrophoresis.
- Gels should be loaded by population. Generally, we load each of two populations on separate halves of the gel with a molecular weight marker in an intervening lane.
- Molecular weight makers on the two outside lanes will help in alignment of bands during the analysis stage.

Day 4 (Photography and Analysis)

- Gels are stained for 30 minutes in electrophoresis buffer containing 0.5 μg/ml ethidium bromide (ETBr). Electrophoresis buffer may be removed from the electrophoresis tank and the ethidium bromide added.

- A 1.4% agarose gel should be fairly firm, but care must be taken not to bend the gel too much as cracking may occur. A sheet of used X-ray film is excellent for lifting and moving gels from place to place. Moistening the film before slipping it under the gel should help.
- *Ethidium bromide is a powerful mutagen and should be handled with extreme care.* Gloves should be worn by anyone handling ETBr. Containers and equipment that are used with ETBr should be labeled as such. Methylene blue, which is non-toxic and non-mutagenic, may be used with visible light as an alternative, but it is less sensitive than ETBr. Another florescent dye (SYBR Green I) is less mutagenic and more sensitive but requires additional filters for photography than those used for ETBr.
- A digitized image may be printed and given to the students for analysis. Overlaying the image with an overhead transparency of a graph may help students identify bands of similar molecular weight.
- We have our students calculate the molecular weights of all the alleles using the molecular weight marker as standards. While this is a good learning exercise and will re-enforce the use of logarithms, it is not a necessary part of the analysis.
- Similarity indices are calculated for each pair both within and between populations, and then average similarity is used to estimate whether or not the populations are subdivided. This superficial analysis is not intended to be statistically sound, but should provide students with an indication of processes occurring at the population level.
- This lab is organized so that it can be completed entirely by the students, taking place over four lab periods. If done this way, Day 3 will require only part of the active time of a 3-hour lab, so that another lab exercise may be accomplished in the remainder of the time. The lab as presented in this handout is accomplished in two lab periods. Prior to the first lab, students collect their insect specimens. In the first lab period, students extract the DNA from their insects and set up the PCR reaction. The instructor runs the PCR, the electrophoresis, and stains the gel between lab meetings. During the second meeting the students analyze the results.

Lab Setup

Instructor's Work Station:

Equipment
1 PCR machine
1 large (24 sample) horizontal gel electrophoresis rig (~ 20cm gel).
1 Boiling water bath
1 life boat for microfuge tubes.

Student Work Station (4 students per group):

Micro pipette (1 μL – 10 μL)
Micropipette Rack
Pipette tips
Indelible lab marker
Ice Bucket

Per Student

Small container for isopod (or other specimen)
Isopod (or other specimen)
1.5 mL microfuge tube containing 500μL 5% Chelix solution
PCR tube containing 20 μL PCR master mix (kept on ice).

Student Outline

Terms: PCR, primer, DNA polymerase, 3'-OH end, amplification, annealing, aliquot, DNA Replication, RAPD, polymorphism.

Objectives:

1. List the chemical components of a PCR amplification reaction.
2. Construct a diagram showing how target DNA replication is achieved in a PCR cycle.
3. Describe the function of the primers in a PCR reaction.
4. Describe how DNA fragments of various sizes are separated and characterized.

Note: It will be useful to review DNA structure, DNA replication, and the PCR reaction in your text.

Introduction

Molecular biological techniques have become very useful in the study of plant and animal ecology and evolution. These techniques are used to study naturally occurring genetic variation, and have greatly facilitated the understanding of many problems in behavioral ecology, population biology and evolutionary biology. Today we will examine two populations of terrestrial isopods ('Pill bugs' or 'Potato bugs' or 'Wood Lice') using one of these molecular techniques, RAPD Analysis. Using this technique, we will see if we are able to detect genetic variation within and between two different populations of these isopods.

The techniques are similar to those used in forensic studies (e.g. DNA fingerprinting as seen on CSI or any other crime drama). The first step is to isolate DNA, the second step is to amplify a portion of the DNA using the Polymerase Chain Reaction and the final step is to examine the amplified sample for polymorphisms (differences within the population and between populations) using gel electrophoresis.

Overview

Polymerase Chain Reaction (PCR): The polymerase chain reaction (PCR) allows the enzymatic amplification of microgram quantities of specific DNA sequences. This technique has greatly facilitated the analysis of sequence variation and has enabled a new level of phylogenetic investigation. PCR also provides a very fast way to screen for variation between a large number of individuals in a study population.

The principle behind PCR is very simple. Short oligonucleotide "primers" are designed so that they will anneal on either side of a 'target' sequence. This sequence can be any size from about 100 base pairs (bp) to 10 kilobase pairs (kb). The target sequence is denatured, the primers annealed, and a copy is produced from each strand using a DNA polymerase (an enzyme that copies a template strand into a new strand of DNA). Then the cycle of denaturing, annealing and extension is repeated so that copies are now made from both original and copy template. The result after numerous repeated cycles is an exponential amplification of the target sequence.

Often, PCR is used to amplify a known sequence of DNA. Thus, the scientists chooses the sequence he or she wants to amplify, then designs and makes primers which will anneal to sequences flanking the sequence of interest. Thus, PCR leads to the amplification of a particular segment of DNA.

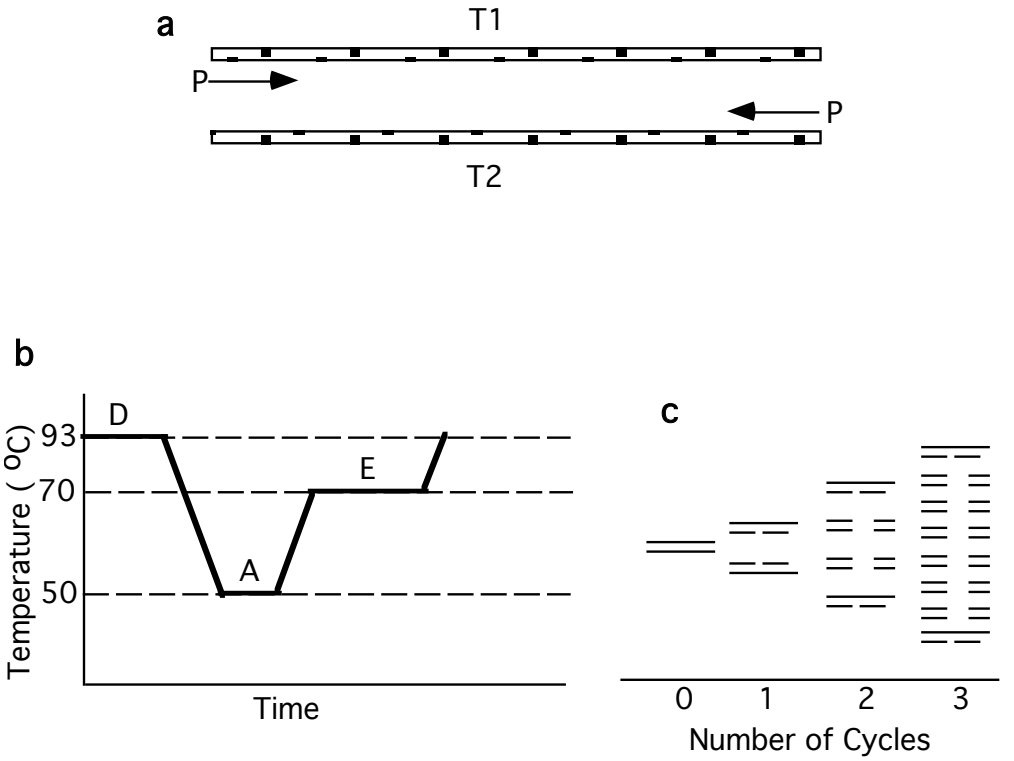


Figure 1. The polymerase chain reaction (PCR). **a)** Primers anneal to denatured template DNA. **b)** The reaction is cycled between denaturing, annealing and extension temperatures. **c)** Repeated cycling results in an exponential amplification of the target sequence.

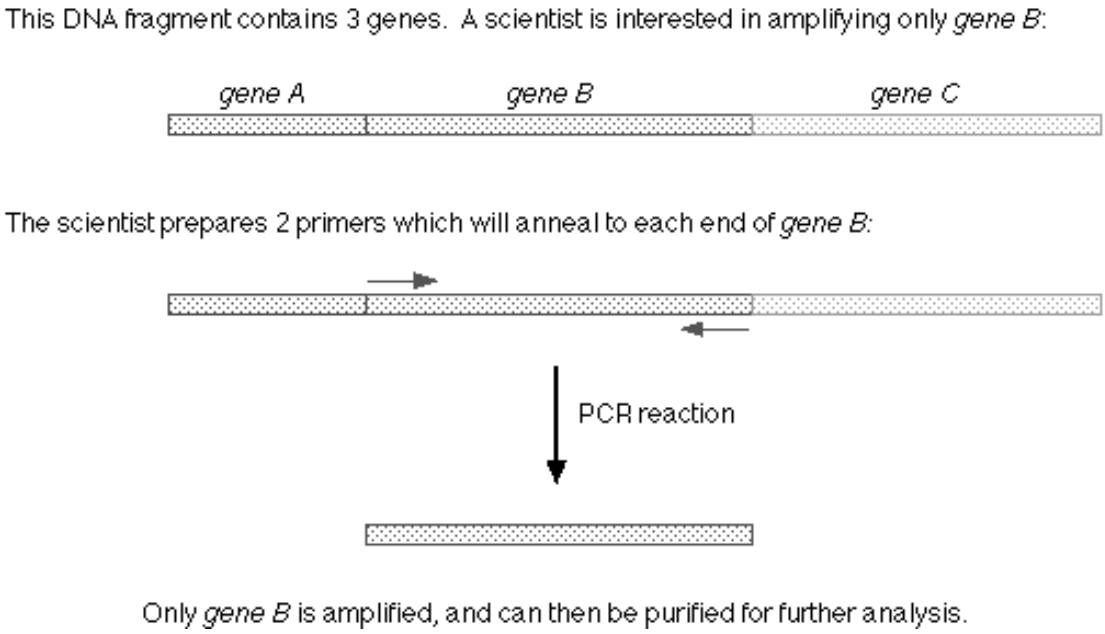


Figure 2. Standard PCR amplification of a specific DNA target.

Remember! In order for PCR to occur:

- The primers must anneal in a particular orientation (such that the 3'-hydroxyl group point towards each other).
- The primers must anneal within a reasonable distance of one another (100 – 10,000 base pairs).

RAPD PCR: RAPD stands for **R**andom **A**mplification of **P**olymorphic **D**NA. RAPD reactions are PCR reactions, but they amplify segments of DNA that are essentially of unknown function to the scientist (random). The scientist designs a primer with an arbitrary sequence. In other words, the scientist simply makes up a 10 base pair sequence (or may have a computer randomly generate a 10 bp sequence), then synthesizes the primer. The scientist then carries out a PCR reaction and runs an agarose gel to see if any DNA segments were amplified in the presence of the arbitrary primer. Figure 3 depicts a RAPD reaction. A large fragment of DNA is used as the template in a PCR reaction containing many copies complimentary to a single arbitrary primer.

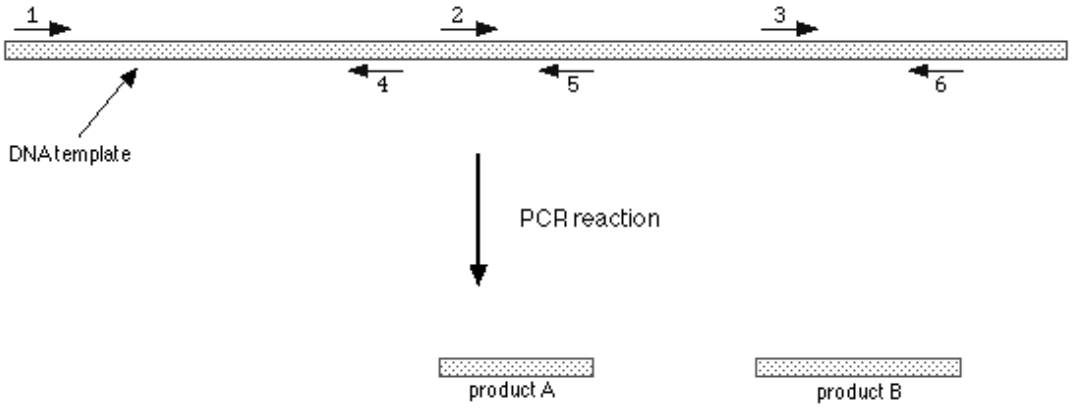


Figure 3. RAPD PCR reaction.

The arrows represent multiple copies of a primer (all primers (arrows) have the same sequence). The direction of the arrow also indicates the direction in which DNA synthesis will occur. The numbers represent locations on the DNA template to which the primers anneal. Primers anneal to sites 1, 2, and 3 on the bottom strand of the DNA template and primers anneal to sites 4, 5, and 6 on the top strand of the DNA template.

In this example, only 2 RAPD PCR products are formed:

1) Product A is produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 2 and 5.
2) Product B is the produced by PCR amplification of the DNA sequence that lies in between the primers bound at positions 3 and 6.

Note that no PCR product is produced by the primers bound at positions 1 and 4 because these primers are too far apart to allow completion of the PCR reaction. Note also that no PCR products are produced by the primers bound at positions 4 and 2 or positions 5 and 3 because these primer pairs are not oriented towards each other.

Finding Differences Between Genomes Using RAPD Analysis: Differences in the RAPD reactions among individuals can be the result of insertions or deletions between primer pairs (changing the length of the DNA fragment being amplified) or from changes of the nucleotide sequence at the primer annealing site (resulting in the loss or formation of a primer site relative to other individuals). Consider the figure

on the page above. If another DNA template (genome) was obtained from a different (yet related) source, there would probably be some differences in the DNA sequence of the two templates. Suppose there was a change in sequence at primer annealing site #2:

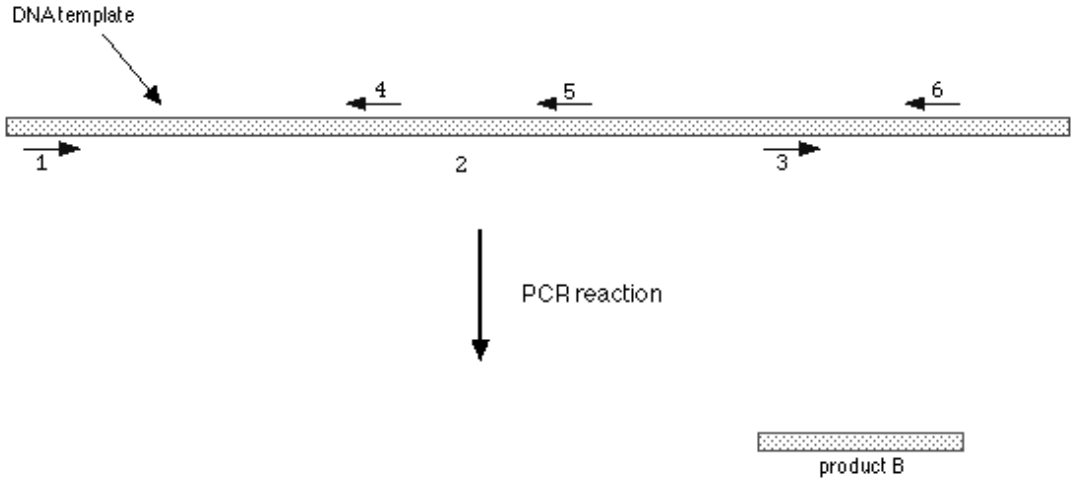
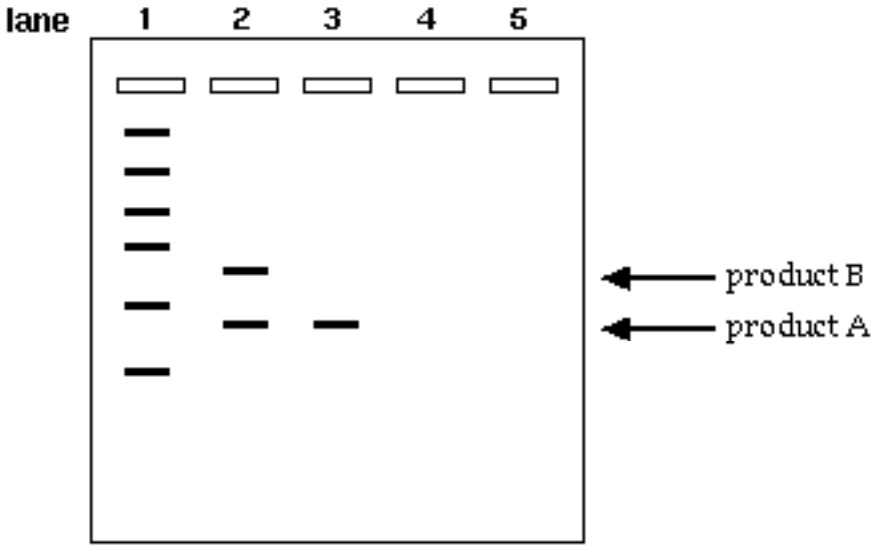


Figure 4. PCR RAPD reaction # 2. In this case the primer is no longer able to anneal to site #2, and thus the PCR product A is not produced. Only product B is produced.

If you were to run the 2 RAPD PCR reactions diagrammed above on an agarose gel, this is what you would see:



lane 1: molecular weight markers

lane 2: RAPD Rxn. #1

lane 3: RAPD Rxn. #2

The advantage of using RAPD technology to examine genetic variation within and between populations is that unlike normal PCR the researcher is not required to know anything about the DNA sequence in the target organism. In other words, knowledge of the particular sequence of nucleotides

being amplified is unnecessary when using this technique. This makes RAPDs an approach useful to any organism from bacteria to mammals. The primers will simply anneal to random segments of the DNA and amplify those. Besides being useful to determine levels of genetic variation within individual species (Maciuszonek, et al. 2005), RAPD technology is also used to uncover evidence of hybridization between species (Stott et al. 2005), and for the identification of stage-specific gene expression during development (Sunagawa and Magae 2005). Disadvantages of RAPDs are that they are dominant genetic markers. This makes it statistically more difficult to perform population genetic analysis as compared to codominant markers (Zhang and Hewitt, 2003, Vignal et al, 2002)

Electrophoresis: The differently sized DNA fragments generated by a PCR reaction can be separated by a technique called agarose gel electrophoresis. The rate of migration of a DNA fragment through a gel is dependent upon four parameters: 1) the agarose concentration in the gel, 2) the voltage applied, 3) the conformation of the DNA (superhelical, nicked circular, or linear), and 4) the molecular size of the DNA fragments. The agarose concentration of the gel and the voltage applied to the gel are readily controlled by the researcher. Because all DNA fragments generated by PCR are linear, size of the DNA fragments is the only remaining variable. More concentrated gels (> 1.5% agarose) are used to separate smaller sized fragments (<2kb). The higher the applied voltage the more rapidly the DNA fragments move but the separation of similarly sized fragments becomes poorer. At high voltages (> 100 v) the gel or the gel mold may melt.

After PCR amplification, the generated DNA fragments are placed in a well in the semi-solid agarose gel. (Instructions for pouring and performing electrophoresis on an agarose gel can be found in Appendix A). An electrical current is applied to the gel and the DNA fragments move a distance through the gel that is inversely proportional to the $\log_{10}$ of their molecular size. Thus, smaller DNA fragments move faster and further from the well than large DNA fragments. Because DNA is negatively charged as a result of its phosphate groups, the fragments always move toward the anode(+) electrode. When DNA standards (fragments of known size) are run on a gel, the distance that each fragment size migrates from the well can be measured and plotted on a graph vs. the $\log_{10}$ of nucleotide pairs of that fragment. (See Figure 1.) The calculation of logarithms can be avoided by the use of semi-log paper.

Visualization of DNA Fragments: Ethidium bromide (EB), a dye which binds to DNA, is used to visualize the DNA fragments in agarose gels. When the gel is viewed under shortwave ultraviolet light (250-300 nm), DNA fluoresces a bright orange-pink color due to the intercalation of the ethidium bromide within the DNA double helix. When ethidium bromide is incorporated in the gel, the DNA fragments become visible during electrophoresis.

CAUTION! Ethidium bromide is a powerful mutagen and should be handled with the utmost caution! Gloves should always be worn when handling gels containing EB. All EB-stained gels, EB-contaminated yellow pipette tips, and EB-contaminated gloves must be discarded in the large white pail labeled "SOLID EB WASTE-, the gel buffer will also contain EB from the gel so the buffer should be disposed of in the white pail labeled LIQUID EB WASTE". These buckets will be disposed of as hazardous wastes.

A photograph of the gel on the UV light box is usually taken to provide a permanent record of the results. Often the distances that the fragments moved are in fact measured from the photograph and not the gel. After the photograph is taken, the DNA fragments in the gel may then be transferred to a membrane for permanent storage (Southern transfer), further purified for sub cloning, sequencing, etc.

Gels can be wrapped in Saran wrap and stored in the refrigerator. In time the DNA bands diffuse and become progressively fuzzier.

Determining size of DNA fragments: Size of unknown DNA fragments (RAPD fragments) is determined by comparing the distance migrated with DNA fragments of known size, the size standards. The fragment sizes of the DNA standards (in kb) are plotted on the Y axis (vertical scale) vs. the distance migrated in mm on the X axis. A "best fit" curve is drawn connecting the different points to generate a standard curve (figure 6). The standard curve tells you the exact relationship between the fragment size and distance migrated for all DNA fragments on a particular gel. (Never use a standard curve from one gel to calculate the size of fragments on another gel.) DNA digestions are run on the same gel as the DNA standards and the distance each DNA fragment moves (in mm) is measured from the well. The distances are then found on the standard curve and the corresponding fragment sizes may be read on the Y axis.

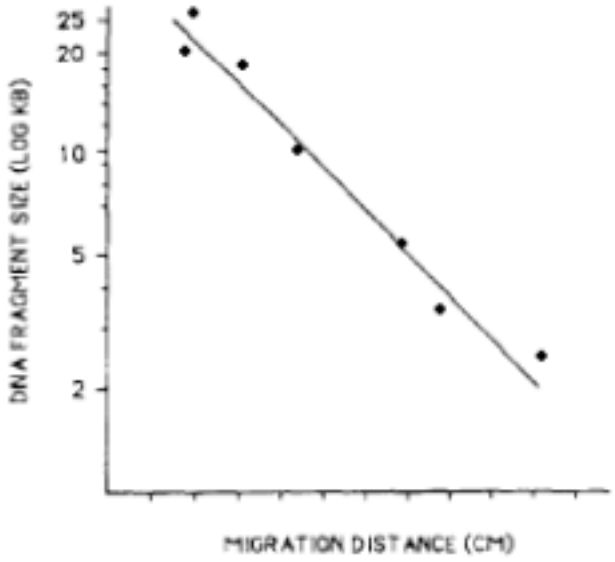


Figure 6. A plot of the distance migrated by DNA fragments in an agarose gel vs. the molecular size of the DNA fragments using semi-log paper, Note the logarithmic scale on the "y" axis but the arithmetic scale on the "x" axis.

Methods

1. Remove a single isopod from the collection jar to a small beaker. Place the beaker in the ice bath until the isopod quits moving.
2. Using a dissecting microscope, remove 4 to 6 legs from the isopod, transferring each leg to a tube of Chelex resin. (Chelex is a resin that binds heavy metal ions that may inhibit PCR reactions).
3. Label your tube with your number and place the tube in the float rack by the boiling water bath.
4. The tubes will be boiled for 15 minutes. This should release DNA from the isopod legs into solution.
5. Remove your tube to your bench top. Carefully remove 5 μL to the PCR reaction tube provided by your instructor.

Note: This tube contains all of the necessary components for making a DNA strand (Primers, nucleotides, buffer, Taq DNA polymerase) other than the template. You will be adding the template.

6. Mix the template and the reaction mix by flicking the tube a couple of times. Place your tube in a microcentrifuge and give it a quick spin to settle the contents to the bottom.
7. Your tubes will be loaded into the PCR Machine where DNA amplification will take place as described above. The resulting fragments will be run on a 1.2% agarose gel. This gel will be photographed and made available to you electronically before the next lab period.

Data Analysis

From the photograph, get the measurement (the distance traveled from the origin) of each of the marker bands and record that measurement in table 1 below.

Table 1. Migration distance of PCR fragments

Mol. Wt.	Distance Migrated
12216	
11198	
10180	
9162	
8144	
7126	
6108	
5090	
4072	
3054	
2036	
1636	
1018	
517	
396	
344	

Variation Within and Between Populations

Now we know the molecular weight of each of the (major) bands in each individual from both populations. We want to determine the similarity between individuals within a population and the similarity of individuals between populations. We are going to make a crude estimate of the average similarity. We will define similarity as the fraction of shared bands for individuals *x* and *y*. It is calculated using the formula below, where n_{xy} equals the number of common fragments in their fingerprint profiles, and n_x and n_y represent the total number of bands exhibited by each of the two individuals.

$$S_{xy} = \frac{2n_{xy}}{n_x + n_y}$$

Average similarity then is the sum of the S*xy* for each pair for within population comparisons divided by the total number of pair wise comparisons. This can be a daunting task. Your instructor will help divide the labor among your class mates. Detailed instructions for performing this calculation can be found in Appendix B.

Acknowledgements

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About the Authors

Bob Sheehy received his Ph.D. in Genetics from the University of Arizona in 1995. He is currently an Associate Professor of Biology at Radford University. He has taught Introductory Biology, Genetics, Human Genetics, Evolutionary Biology, Molecular Biology, Molecular Ecology, Ornithology and Conservation Biology. His research interests include Phylogeny reconstruction and character evolution in the Falconiformes and population genetics of sub-divided populations.

Judy Guinan received her Ph.D. in Ecology from the University of Georgia in 2000. She was awarded UGA's Excellence in Teaching award in 1999. She is an Assistant Professor of Biology and Director of the Radford University Environmental Center. She has taught Introductory Biology, Environmental Biology, Conservation Biology, Ecology, Evolution, and Animal Behavior, and her research interests include sex ratio variation and the reproductive strategies of females, including those of parasitoid wasps.

Appendix A: How to Pour and Perform Electrophoresis on an Agarose Gel

Agarose gels are like Jell-O. They have two main ingredients, a buffer and agarose (a highly refined form of agar). Agar is the same substance that keeps your commercial ice cream smooth! You will be using gels made with one of two kinds of buffer, TBE (Tris-Borate-EDTA) or TAE (Tris-acetate-EDTA). The percentage gel (w/v) will vary depending on the size of molecules we are trying to resolve. RAPD markers are relatively small and so we will be using a higher percentage of Agarose. A good starting place is 1.2% (remember that a 1% w/v gel is 1 g agarose in 100 ml buffer). Before making the gel set up the casting tray and comb (as described below) and, using a graduated cylinder measure the volume of gel needed.

1. Tape the ends of a gel mold and make sure some of the tape wraps around the bottom of the mold by 1 - 2 mm. Choose the appropriate comb(s) and make sure it looks clean.
2. Make the appropriate volume of gel in a 250 - 500 mL flask, cover it with aluminum foil (yes it is safe), and microwave for 5 minute on medium power (a good starting time).
3. Visually check to see that all the agarose has melted. Unmelted agarose looks like tiny refractive lenses floating around. If not completely melted, nuke it a little longer. Try 1 minute.
4. Allow the gel to cool a bit; you may hasten this by running cold water over it but do NOT let it cool too much. Alternatively, you may place the flask in a 55°C water bath to keep the gel liquefied.
5. Add the appropriate volume of ethidium bromide (EtBr; 10 mg/mL stock) to achieve a final concentration of 0.05mg/mL. **NOTE: EtBr is a *known mutagen,* so wear gloves!!!**

Sample calculation:

Let's say you are pouring a 200 mL gel. You want a final EtBr concentration of 0.5 mg/mL and your stock solution is 10 mg/mL. Remember that micro (m) = 10^{-6} and milli (m) = 10^{-3}:

We can use the equation: $V_1C_1 = V_2C_2$

V_1 = volume of EtBr we need to add to the gel to make a solution with a final concentration of 0.5 mg/mL,

V_2 = Volume of the Gel (200 mL)

C_1 = the concentration of EtBr in the stock Solution (10 mg/mL)

C_2 = desired final concentration (0.5 mg/mL)

V_1 x 10 mg/mL = 200 ml x 0.5 μg/mL x ?

$$v_1 = \frac{200mL * 0.5\mu g/mL}{10mg/mL} = \frac{200mL * 5^{-7}\ \mu g/mL}{10mg/mL} = \frac{10^{-4}\,g}{10^{-3}\,g/mL} = 0.01\text{mL} = 10\mu\text{L}$$

6. Carefully pour the gel into the casting try being careful not to introduce bubbles. If bubbles are seen in the gel use a pipette tip to push the bubbles to the end of the gel away from the comb
7. Allow the gel to cool until it turns translucent white. The gel is ready to run, as soon as you pull off the tape,
8. Remove the comb, and submerge it in 1 X buffer. The running buffer should just cover the wells of the gel.
9. Hook up the electrodes with the negative lead connected at the end of the gel box closest to the wells. Turn on the power and make sure there is a connection. You should see approximately twice as many bubbles coming off of the electrode on the end of the gel towards the wells. (Why is there a difference?)
10. Generally the slower the gel is run the better the resolution. We will run the gel at 25 volts overnight. If TAE buffer is used as the running buffer, we will circulate the buffer to prevent changes in pH over the course of electrophoresis.

Appendix B: Calculation of the Average Similarity Within and Among Populations

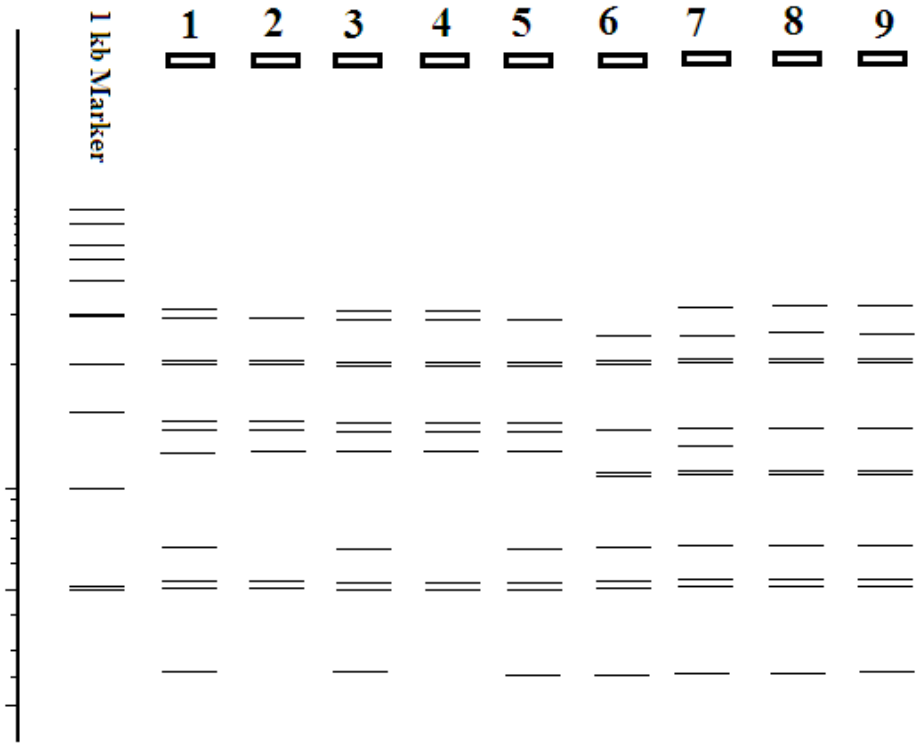

Figure 7. An ideogram of a RAPD gel.

In the farthest left lane of Figure 7 are the DNA markers that may be used to determine the approximate size of DNA fragments amplified by PCR. Lanes 1 – 5 are RAPD amplifications from population A while lanes 6 – 9 contain RAPDs from population B. We want to determine the average similarity of band sharing within each population and then between populations. We will start by determining the number of bands in each individual:

1) Count and record the number of bands that appear in each lane.

Lane	1	2	3	4	5	6	7	8	9
Population	A_1	A_2	A_3	A_4	A_5	B_1	B_2	B_3	B_4
# of Bands	11	8	11	9	10	10	12	11	11

2) Now, determine the number of bands ***shared*** in each pair-wise comparison. This value we will denote n_{xy}

	A_1	A_2	A_3	A_4	A_5	B_1	B_2	B_3	B_4
A_1									
A_2	8								
A_3	11	8							
A_4	9	8	9						
A_5	10	8	10	8					
B_1	7	5	7	5	7				
B_2	8	7	8	7	7	10			
B_3	7	6	8	6	7	10	11		
B_4	7	5	8	6	7	10	11	11	

3) Now we determine the frequency of those bands that are shared between Pairs of individuals. We will call this value Sxy. This value is determined by the formula:

$$S_{xy} = \frac{2n_{xy}}{n_x + n_y}$$

For example, the number of bands shared between individuals A1 and A2 = 8 (this value is n_{xy}, A1 has 11 (this value is n_x) bands and A2 has 8 bands (this value is n_y) therefore, the similarity between A1 and A2 =:

$$S_{xy} = \frac{2n_{xy}}{n_x + n_y} = \frac{2*8}{11+8} = \frac{16}{19} = 0.84$$

	A_1	A_2	A_3	A_4	A_5	B_1	B_2	B_3	B_4
A_1									
A_2	0.840								
A_3	1.000	0.842							
A_4	0.947	0.941	0.947						
A_5	0.952	0.888	0.952	0.842					
B_1	0.666	0.555	0.666	0.526	0.700				
B_2	0.696	0.700	0.696	0.666	0.636	0.909			
B_3	0.636	0.632	0.727	0.600	0.666	0.952	0.957		
B_4	0.636	0.526	0.727	0.600	0.666	0.952	0.957	1.00	

4) We now have pair-wise comparisons within populations (light and and darkly shaded gray in the table above) and between populations (white background). The next step is to determine the average similarity within and among populations. For each group determine the average similarity.

Within for population A:
(0.840 + 1 + 0.947 + 0.952 + 0.842 + 0.941 + 0.888 + 0.947 + 0.952 + 0.842)/10 = 0.915

Average similarity within population B:

(0.909 + 0.952 + 0.952 + 0.957 + 0.957 + 1.00)/6 = 0.955

Average similarity between populations:

(0.666 + 0.696 + 0.636 + 0.636 + 0.555 + 0.700 + 0.632 + 0.526 + 0.666 + 0.696 + 0.727 + 0.727 + 0.526 + 0.666 + 0.600 + 0.600 + 0.700 + 0. 636 + 0.666 + 0.666)/20 = 0.646

5) We will now calculate the standard error within and among populations. This is done for each population and for the between population comparisons. This will be done in seven steps.

- Calculate each measurement's deviation from the mean (mean minus the individual pair-wise comparison.
- Square each deviation from the mean (squared negatives become positive).
- Sum the squared deviations.
- Divide the sum from above by one less than the sample size (n-1).
- Take the square root of the number from above. This gives you the "standard deviation (S.D.)."
- Divide the standard deviation by the square root of the sample size (n). This gives you the "standard error"

Example:

Pairwise Comparison	Similarity	Deviation (m-i)	Squared deviation $(m-i)^2$
A_1A_2	0.840	0.075	0.0056
A_1A_3	1.000	-0.085	0.0072
A_1A_4	0.947	-0.032	0.0010
A_1A_5	0.952	-0.037	0.0014
A_2A_3	0.842	0.073	0.0053
A_2A_4	0.941	-0.026	0.0007
A_2A_5	0.888	0.027	0.0007
A_3A_4	0.947	-0.032	0.0010
A_3A_5	0.952	-0.037	0.0014
A_4A_5	0.842	0.073	0.0053
n=10	Mean m =0.915		Sum of squared deviations $\sum (m-i)^2 = 0.0296$

Divide by the number of measurements-1. $\sum (m-i)^2 /(n-1) = 0.0296/9 = 0.00329$

Standard deviation = square root of $\sum (m-i)^2 /(n-1) = \sqrt{0.00329} = 0.057$

Standard error = standard deviation/$\sqrt{n}$ = 0.057/$\sqrt{10}$ = 0.057/3.162 = 0.018

Mean similarity ± 1SE = 0.915 ± 0.018 or 0.897 to 0.933.

We calculate this for each population and for the between populations. If the standard errors of between population average similarity does not overlap with the average similarity within populations then we can conclude that there are two distinct populations. If the standard error of the among population average similarity does overlap with the within population similarity than either what we called, based on collection, two populations are either interbreeding (and thus one population) or the populations have not diverged significantly to detect a difference. The within and among average similarity ($\overline{S}$) ± 1SE are:

$\overline{S}$ ± 1SE for Population A = 0.897 to 0.933
$\overline{S}$ ± 1SE for Population B = 0.943 to 0.966
$\overline{S}$ ± 1SE between populations A and B = 0.633 to 0.660

Since the average similarity of the between population comparisons does not overlap with either of the within average similarity then we can conclude that these are different populations.

Keep in mind that when we calculate mean and standard error we are assuming that each sample is independent of other samples. Since we are using pair-wise comparisons and we use the same sample multiple times (A1A2 A1A3, A2A3 etc.) our samples are not truly independent. To make this statistically valid we would have to take these non-independent samples into account with for example the Bonferroni Correction, which is beyond the scope of this lab.

Appendix C: Checklist For Equipment and Reagents

- agarose: (low EEO) Fisher #BP160-100 or equivalent
- 20X TAE or 5X TBE:
- micropipettes (1 – 10 μL for students and 1 – 10 μL, 5-50 μL and 50 – 200 μL for instructors):
- yellow tips
- barrier tips (1 – 10 μL size)
- Thermo stable DNA polymerase (Taq or equivalent)
- Nucleotides (dNTP's)
- Tweezers
- microfuge tubes: (500 μL) (1.5 mL) for preparation of stock solutions
- microfuge tubes: (1.5 mL) for preparation of stock solutions
- photo setup and UV box: Polaroid is good
- microwave: standard, should hold 250 mL flasks
- 250 mL flasks
- hot gloves
- ethidium bromide solution: (10mg/mL stock)
- balance: (0.01 - 10 gram range minimum)
- weigh paper
- small spatulas
- distilled water
- graduated cylinders: 1 liter and 100 mL
- PCR machine that holds 500 μL thin walled microfuge tubes
- VWR lab markers: (black; extra fine tips) #52877-150
- microfuge tube racks
- gel molds, boxes and power supplies
- crushed ice and ice buckets
- microfuge
- -20° C freezer (preferably frost free) and +4° C refrigerator

Appendix D: Recipes for Reagents

20X TAE Buffer (pH 7.8)

Volume/Concentration	20x = 1L
0.08M Tris Base	96.8 g
Glacial acetic acid	22.8 mL
0.05 M EDTA	100 mL 0.5M EDTA pH 8.0
dH_2O	750 mL

Adjust pH to 7.8
Adjust volume to 1 liter

10x TBE Buffer (Tris-Borate-EDTA)

108 gm	Tris base
55 gm	Boric acid
9.3 gm	Na4EDTA

Add ddH2O to 1 liter.
The pH is 8.3 and requires no adjustment

dNTP mixture (1-400μl batch, 10mM)

10μl of each 100mM dNTP stock solution (dATP, dCTP, dGTP, dTTP)
360μl autoclaved distilled water (best to use a thawed frozen tube of water)
mix gently, store in freezer [ideally make 5 at a time]

PCR Master Mix for 25 µl Reaction:

Ingredient	Final Concentration	Volume	Number Num Rx + 1	Total Volume
10x dNTP	1 mM	2.5 µL		
10x Primer	0.5 µM	2.5 µL		
10x Buffer	1x	2.5 µL		
Taq DNA polymerase	1 unit/Rx			
H_2O				
Total		20 µL		

Primers store at -20° C

Taq DNA polymerase store at -20° C

DNA ladder plus tracking dye (180µL, 1 tube)
160µL H_20
10µL 250 bp (or other) molecular weight-sizing ladder stock
10µL tracking dye stock mix well, use 10µl as standard in 2+ wells of final gel, to size molecular markers store in freezer

10X Buffer (with loading dye):

Final Concentration	Amount from stock
500 mM Tris pH 8.3	2.5 mL Tris pH 8.3 (2 M stock)
2.5 mg/ml BSA (Bovine Serum Albumin)	0.5 mL BSA (50 mg/mL)
20% Sucrose	5 mL 40% sucrose
1 mM Cresol Red	1.0 mL10 mM Cresol Red (sigma 114472)
50 mM $MgCl_2$	500 µL 1 M $MgCl_2$
	500 µL H_2O

Ethidium bromide stock (10 mL, 10 mg/mL)
0.1g ethidium bromide
10 mL distilled H_20
Mix in centrifuge tube. Store at room temperature wrapped in aluminum foil

TE Buffer (250mL)
0.303g Tris
0.093g EDTA
250mL distilled H_20
pH to 7.5 with HCl
store in refrigerator