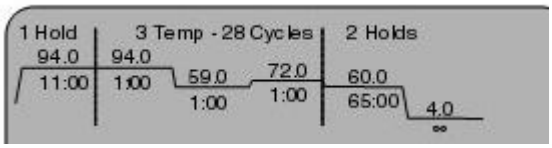
Molecular Markers in Chestnut

Introduction: we are screening the DNA of our sample to locate short segments of DNA that are different between individuals that we can use as markers that indicate whether two individuals are related, identical (also called clonal) or unrelated. We have to verify that the markers are nuclear (that is, on chromosomes that segregate and not on the chloroplast or mitochondrial genomes that are maternally inherited). Clearly we also used a chloroplast marker earlier, so the marker we select depends on the question being asked. Each segregating marker only tells you about the inheritance of its immediate location, so if we want to follow the inheritance of a particular characteristic (like disease resistance, or a flower color) we need to identify a marker that is close to, if not actually part of, the gene of interest. During the screening process the goal is to identify PCR primers that yield lots of markers. Some will then be shared by different individuals and some will be different, giving us a way to estimate the closeness of the relationship. We will have used three types of markers by the time we are done: the chloroplast marker that tells you if you have a maternal line of American or Chinese chestnuts, microsatellite markers that have length differences between individuals, and Randomly Amplified Polymorphic DNA (RAPD) markers that give a plus/minus difference between individuals. The chloroplast and microsatellite markers each target one specific locus, while the RAPD procedure uses a short piece of DNA (only 10 bases long) as the primer to the PCR reaction – it will bind in multiple locations and give us products from each location when two binding sites are close enough together.

PCR Details: because the primer is short, we won't use a very high temperature for the annealing part of the PCR reaction with the RAPDs. The final extension is at the temperature that the Taq polymerase enzyme prefers, 72C. The PCR requires that we melt the two strands of genomic DNA target apart with high heat and then quickly cool the mixture to the temperature where the primer can bind the DNA, after which we heat slowly back up to the temperature that Taq polymerase likes best. We use a specialized heat block called a thermocycler to carry out the temperature transitions. The pattern shown in the picture below is the 'cycling profile' – we program this into the instrument before we put in the samples. Note that if we do 30 cycles with these times this will take ~3 hours (times are shown in minutes). Since we don't want to wait around to take the samples out, the instrument can cool to 4C and hold that temperature indefinitely, so the samples will be safe until the following morning.



The RAPD primers serve as both forward and reverse primers, so you only need to add one primer per reaction, the chloroplast and microsatellite loci need separate forward and reverse primers (but we have mixed them together for you).

PCR reactions are usually run in small (200ul) thin-walled tubes – thin so that the heat transfers quickly. Normally you do not put more that 50ul into one of these tubes (we are using 25ul). Since you are heating the reaction almost to boiling, the solution should evaporate to the top of

the tube. However the lid is heated to just above 100C, which forces anything that evaporates to move even faster in the opposite direction (down to the bottom of the tube). This way you don't have to add a layer of oil or wax to the top of your reaction to prevent evaporation, which was really inconvenient.

Each team will set up 5 PCR reactions for each sample on each of two days, using the chestnut DNA you have purified and 4 different primer mixes as well as one negative control (where you add water or TE buffer instead of primers). On the first day we will screen 4 RAPD primers, and on the second day we will screen 4 microsatellite primer pairs and one negative control. You will run 5ul of each 25ul PCR reaction on a 1.5% agarose gel in order to analyze the results. Screening means we don'tknow if the reaction will yield bands or not, so don't be surprised if some of your reactions yield a smear instead of a set of clear bands.

Stage 1: RAPD Markers

Each individual reaction has a final volume of 25ul. You will be given a Master Mix that contains the buffer and 4mM Magnesium, the nucleotides, some Taq polymerase and a small amount of a protein called BSA which often improves the efficiency of PCR reactions. This Master Mix has been made up ahead of time, so you use 20ul of it. You then have 5 ul of volume for adding your DNA and your primers. The primers are at 10uM, and you will add 2ul of each primer mix to one specific PCR tube. That means you have 3ul to add your DNA. You should add 150ng of DNA to your reactions – that means your DNA has to be at a concentration of 50ng/ul, the concentration you made for the chloroplast test – you should have more of this stock available. If not prepare as indicated in the first step.

1. Make 100ul of a dilution of your genomic DNA so that the final concentration is 50ng/ul
    a. For example, if your DNA measured 300ng/ul on the spectrophotometer you would say that 100ul (50ng/ul) = Xul (300ng/ul)
    or 5000/300 = Xul which means you will need to use 16.7ul of your genomic DNA sample.
    b. Since you want your final volume to be 100ul you will add
    100-16.7 = 83.3ul of 1X TE buffer.
    c. Vortex the DNA and TE buffer, then quick-spin in the minifuge – now you have your Stock Genomic DNA.
2. Place 5 PCR tubes in a rack on ice.
    a. In each tube micropipette 20ul of Master Mix
    b. In each tube micropipette 3ul of diluted DNA
    c. In each tube micropipette 2ul of ONE of the primers. You will have more tubes than primers – in the last tube pipette 2ul of water.
    d. Label the tubes

e. Carry the tubes to the PCR machine labeled RAPD – there will be a rack in an ice bucket next to it, put your CP reaction tube and your H2O reaction tube in that rack – the instructor will put the tubes in the PCR machine and start the profile once all the students have completed their reactions. Carry the remaining 3 tubes to the PCR machine labeled 'RAPD' and put the tubes in the rack in the ice bucket there.
    i. Do check that your labels do not match anyone else's – you are going to need to reclaim these reactions and run 9ul of each PCR reaction on an agarose gel.

3. The second set of reactions will use 4 sets of microsatellite primers – these use the same Master Mix, and we will use the chloroplast thermocycling protocol.

Note: While the samples are thermocycling, pour a 1.5% agarose gel.

1. Be sure to select a casting tray and comb that will allow you to run 5 reactions for 2 samples = 10 lanes +2 marker lanes or 12 lanes total.
2. The only difference in the procedure compared to earlier protocols for these gels is that you will need to add 1.5gm of agarose to 100ml of 1X TBE buffer.
3. You will load 9ul of each sample + 3ul of 4X Gel Loading Buffer per lane, and 3ul of the Bionexus Hi-Lo All-Purpose ladder + 3ul of water in the two end lanes.
4. Electrophorese at 50V for 2-3 hours.

Components of the PCR reaction

V_t = 25 ul (or total Volume = 25 microliters).

1.0uM primer	Final concentration, see about calculations below
0.2mM of each dNTP	" "
4mM MgSO4	" "
1X PCR buffer.	" "
30 ng DNA	Total amount
2.0Unit Taq DNA polymerase	Total amount
10ug of BSA	" "
Molecular-biology grade water to bring the final volume to 25ul	

The ***Master Mix*** idea: if you are adding almost the same thing to every reaction, you can make one solution and distribute it equally across the tubes. Then you add the one thing that is different (here, the primer).
Why do this? It limits the pipetting you have to do, which is good but it also reduces the number of places where you can make errors.

Note on Master Mixes: Since there are errors associated with pipetting small volumes, the rule is to always make at least 10% (multiply the volume by 0.1) more volume than you think you need. If we need 150ul of solution, we multiple 175ul * 0.1 = 7ul more, so we make 175ul + 17 ul = 194 ul. Since I like round numbers for doing the calculations, I will round this up to 200ul, which is what I would need to do 8 reactions.

Calculations for Master Mix for 5 reactions of 25ul for 9 teams – total volume is 1000ul, to be made up to 1250ul after adding DNA and primers.

The primer stock is at 10uM .

The Buffer stock is at 10X

The enzyme is supplied at 5 Units per microliter.

The dNTP stock is at 10mM, but this is for all 4 types combined (A,T,C,G) so each individual one is 2.5mM in the stock.

The MgSO4 is supplied at 50mM

The BSA is supplied at 10mg/ml

Component	Stock Concentration in tube in ice bucket	Vol to use (ul) for Master Mix (MM)
Water	NA	605
10X PCR buffer or 5X buffer	10X	125
dNTP mix	10mM (2.5mM each)	100
MgSO4	50mM	100
BSA	10mg/ml	50
Taq pol.	5U/ul	20

Leave the PCR tubes on ice while you set up the thermocycler with the following profile:

RAPD PCR Thermocycling Profile

Melt
- 98C for 4 min

Cycle 10 times
- 98C 30sec
- 30 30sec
- 68C 1 min

Cycle 35 times
- 98C 30sec
- 35C 30sec
- 68C 1 min

Finishing
- 72C 7 min
- 4C hold

Place the tubes in the thermocycler and start the program.

Chloroplast and Microsatellite Thermocycling protocol

Melt
- 95C 4min

Cycle 35 times
- 92C 20sec
- 48C 20 sec
- 72C 20sec

72C 7 min
4C Hold.

To analyze the samples, remove 9ul of each PCR reaction from the tube, add 3ul of 4X loading dye.
Visualize on 1.5% agarose gels run in 1X TBE buffer. Use DNA size markers that range from 100bp to 2000 bp.