# Molecular Markers in Chestnut

Introduction: we are using bits of DNA as the markers to say whether two types of chestnut tree are the same. Individual trees of the same species will be unique in some locations, so the type of marker we select depends on the question being asked. Here, we want to have lots of markers, so that some will be the same and some will be different, giving a way to estimate the relationship. We will use three types of markers: a chloroplast marker that tells you if you have a maternal line of American or Chinese chestnuts, microsatellite markers that have length differences between the two types of species, and Randomly Amplified Polymorphic DNA (RAPD) markers that give a plus/minus difference between the two species. The chloroplast and microsatellite markers target one specific locus, while the RAPD procedure uses a short piece of DNA (only 10 bases long) as a primer to the PCR reaction – it will beind in multiple locations and give us several bands. 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. Because PCR requires that we melt the two strands of DNA apart with high heat and then quickly cool the mixture to the temperature where the primer can bind the DNA, we then heat (‘ramp’) 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 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. Since we don’t want to wait 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).

Each team will set up 5 PCR reactions on each of two days, using the chestnut DNA and 4 different primer mixes and one negative control (add water instead of primers). On the first day we will do the chloroplast test and 3 RAPD primers (we will use 2 different PCR machines since the profiles are very different), along with one control. On the second day we will will use 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.

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 a little more than the base, 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, which was really inconvenient.

Chloroplast (CP) 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 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 30ng of DNA to your reactions – that means your DNA has to be at a concentration of 10ng/ul. So step 1 is to dilute your DNA.

1. Make 100ul of a dilution of your genomic DNA so that the final concentration is 10ng/ul
	1. For example, if your DNA measured 300ng/ul on the spectrophotometer you would say that 100ul (10ng/ul) = Xul (300ng/ul) or 1000/300 = Xul which means you will need to use 3.3ul of your genomic DNA sample. Since you want your final volume to be 100ul you will add 100-3.3 = 96.7ul of 1X TE buffer.
	2. Vortex the DNA and TE buffer, then quick-spin in the minifuge – now you have your diluted sample
2. Place 5 PCR tubes in a rack on ice.
	1. In each tube micropipette 20ul of Master Mix
	2. In each tube micropipette 3ul of diluted DNA
	3. 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.
	4. Label the tubes
	5. Carry the tubes to the PCR machine labeled ‘CP’ – 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.
		1. Do check that your labels do not match anyone else’s – you are going to need to reclaim these reactions and run 5ul of each 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.

**Components of the PCR reaction**

Vt = 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.

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| **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 50mMThe 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 5ul of each PCR reaction from the tube, add 1.5ul 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.