Lab 6: Long-amplicon coverage of the Chloroplast genome: Stage 1 for sequencing.

1-2 days

Introduction:

We are going to cover the chloroplast genome – capture all of the sequence using overlapping ~15,000bp (kbp) PCR amplicons. Why overlapping? Because we want to be able to know the order in which to arrange the data when we have it, the overlaps show us what fits together. Why amplicons? We could isolate the chloroplasts away from the rest of the cell, using a sucrose gradient, and then lyse the chloroplasts and purify the chloroplast genome. However, the sucrose gradients are tricky to run, very messy and time consuming. Since there is a Chinese chestnut chloroplast genome published, we have a good idea about the majority of the sequence. Of course sometimes there might be sequence variation right where I put a primer, and the PCR might either be weak or ineffective – this means we may have to go through a couple of problem solving steps before we are ready to sequence. This is a common factor when designing libraries for sequencing from new species.

PCR reactions can be optimized to give various length products – mostly we use it for shorter (150 – 500bp) products, but some of the thermostable DNA polymerases are capable of making much longer products. You do have to select a polymerase that processes well, and change the thermocycler's profile so that there is sufficient elongation time to produce the desired length – that time depends on the processing rate of the polymerase. Because the extension part of the reaction takes quiet a long time (over-night), we will set the reactions up today and look at the products on gels tomorrow.

Please note that I will have each team test a couple of the primer pairs on both of their samples. These will be done in separate tubes, not all together (for some PCR reactions this is done, it is called a multiplex reaction).

Tomorrow we are going to run 7ul of each PCR product on 0.8% agarose gels, in order to make sure that each sample gives at least one product and only one product, of the expected size.

Protocol

Materials, Reagents and Equipment

1. 1 x 1.5 ml microfuge tube with 120ul of 2X Kapa MyFi PCR mixture – enough for 4 x 50ul reactions.
2. 4 PCR Tubes with 2 pairs of Cp-Amplicon primers (10uM each, you will test your 2 samples with the 2 pairs of primers)
3. 1 x 1.5 ml microfuge tube with molecular-biology grade water (MB-H_2O).
4. Benchtop cooler, with capless 0.5ml tubes used as holders for 4 PCR tubes (with caps).
5. One PCR machine, programmed for long amplicons, with an ice bucket containing a PCR tube rack.

Step 1: Set up PCR reactions

1. Previously you have made a stock DNA solution that is 50ng/ul. You will use some of that DNA stock solution here.
    a. You will add 200ng per PCR tube, which is 4ul.
2. Your *Master Mix* tube contains 120ul of a 2X Kapa MiFi PCR solution. Your PCR tubes contain 5ul of forward and reverse primers.
3. Set up the tubes as follows: To each PCR tube add
    a. 2X PCR MyFi Master Mix
        i. Add 25ul to each PCR tube.
        ii. Use a fresh tip every time!
    b. The DNA
        i. Add 4ul of the DNA stock solution to each PCR tube
        ii. Use a fresh tip every time.
    c. Add water
        i. Add 21ul of molecular-biology grade water (MB-H2O) to each PCR tube.
        ii. Use a fresh tip every time.
    d. Cap the tubes and label them
    e. Put the tubes in the cooler next to the PCR machine – when all of the samples are ready we will heat the machine to 104C, insert the tubes and close the lid.
    f. Wait 8 hours.

Thermocycling Profile - should be named 'Cp15K'. You can check that it looks like this:

95C 60s

30Cycles:

95C 15s
60C 30s
68C 15min

1 Cycle:

68C 7 minutes
Hold 4C*

4. On Friday - run 10ul of the PCR product on a 0.8% agarose gel with DNA ladders that are 15,000bp long so you can make sure you got the expected size of product
    a. I will pour this gel so we can load the samples first thing Friday morning, we will want to see which reactions worked and which did not by the end of the day.