Molecular Systematics MSc module practical session.

Practical sessions run by Mrs Andie Hall, Ms Alex Aitken and Mr Steve Russell.

Introduction.

This module’s series of laboratory and computer practicals is designed to provide initial experience in some routine laboratory techniques used for generating and analysing molecular sequence data.

- Extraction and purification of genomic DNA using a commercial kit
- Use of the nanodrop spectrophotometer to analyse DNA samples
- Amplification of specific genes by the polymerase chain reaction (PCR)
- Purification of PCR products
- Automated DNA sequencing reactions
- DNA sequence editing
- DNA sequence alignment
- Phylogenetic reconstruction from aligned sequence data

The practicals for this module will cover the entire process from whole organism to phylogenetic tree. In laboratory practicals you will be extracting the DNA from different families of beetle and amplifying and sequencing three genes - the mitochondrial protein-coding cytochrome oxidase I gene (COI) involved in the mitochondrial respiration chain, the mitochondrial SSU rRNA gene 16s and the nuclear LSU rRNA gene 28s.

Because of time constraints the normal sequence of events for this practical as listed above may not be adhered to, and in the event that the experimental procedures do not go according to the plan, alternative components and data may be provided.

From start to finish the whole process involves:

1. specimen preparation
2. DNA extraction
3. DNA purification
4. DNA concentration and purity analysis
5. Polymerase chain reaction (PCR)
6. PCR product analysis (agarose gel electrophoresis)
7. PCR clean-up
8. sequencing reaction
9. sequencing reaction clean-up
10. automated sequencing
11. data acquisition
12. data analysis

Due to time constraints and lab conditions, you will perform all these steps except 7 – 11; these will be demonstrated for you.
**Basic Timetable**

**Thursday 20th Jan 10.00 – 17.00**

Group divided into 5 groups of 4.

Each group is provided with 10 Specimens and 10 microfuge tubes.

Students will prepare the specimens and place them in the tubes.

Students will prepare the digestion mix and add this to the specimens. This will be incubated over Lunch.

The students will then process the extraction, collecting 2 elutions.

In groups, Check the gDNA using a nanodrop spectrophotometer.

Recover the voucher specimens.

**Friday 21st Jan 10.00 – 17.00**

The groups will set up 3 reactions for each of their samples, plus one negative control per gene = total 33 reactions per group.

The PCR will run over lunch

Each group will make an agarose gel.

Students will load some of each of their PCR product on their gels

[The loading of samples onto agarose gels can be awkward if you have never done it before, so you have spare wells to practice on.]

Your successful PCR product will be cleaned by the MBU sequencing facility afterwards.

**Monday 24th Jan 10.00 – 17.00**

In the morning you will be taken to visit the MBU sequencing facility, where PCR product cleanup, sequencing reaction set up and sequencing will be demonstrated and explained.

Afternoon: Compilation of your own sequence data with Sequencher (Lower Bridge room)
Laboratory Health and Safety

Some of the reagents you will be using are toxic and PCR reactions are easily contaminated with external DNA from the environment, so it is important that you wear lab coats which are done up, and gloves whenever you are in the laboratory to protect both you and the samples. Particular hazards and associated risks will be explained to you.

Any waste solutions from the experimental process should be sealed in tubes and stored on the bench for collection and disposal. No liquids from these protocols should be poured into the sinks.

Empty tubes and pipette tips should be placed in the large beakers on the bench for disposal.

Gloves, paper towels etc should be placed in the yellow sacks located under the bench.

Wash your hands before leaving the laboratory.

**NO EATING, DRINKING, SMOKING, OR CHEWING GUM IN THE LABORATORY.**

Use of micropipettors.

- Pipettes are very delicate, expensive and liable to contamination.
- Never touch pipettors with bare hands.
- Never touch the barrel of the pipettor.
- Do not drop them onto the bench – place them carefully.
- Maximum pipette volume is shown on the thumb knob – NEVER force pipettes beyond their stated maximum.

<table>
<thead>
<tr>
<th>TIP TYPE TO USE</th>
<th>MAXIMUM VOLUME</th>
<th>MINIMUM VOLUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>Tiny clear</td>
<td>2µl</td>
</tr>
<tr>
<td>P10</td>
<td>Tiny clear</td>
<td>10µl</td>
</tr>
<tr>
<td>P20</td>
<td>Medium clear</td>
<td>20µl</td>
</tr>
<tr>
<td>P200</td>
<td>Medium clear</td>
<td>200µl</td>
</tr>
<tr>
<td>P1000</td>
<td>Large clear</td>
<td>1000µl</td>
</tr>
</tbody>
</table>

- Always adjust the volume by a downwards turn from a larger volume – i.e. if you need to raise the volume set, go past your desired volume and then adjust back down to what you need (e.g. the pipettor is set on 56µl and you need 124µl. Wind the pipette up to about 140µl then adjust to 124µl). This gives greater accuracy.
- There are different models available – the volume adjustment is either made using the thumb knob or the internal collar.

**Good lab practice**
Allow all reagents to thaw before use.
Vortex all solutions before use.
Always put tubes into a microfuge for a quick spin before opening, to make sure no liquid is left on the lid.
When using a microfuge, spread out your tubes to keep them balanced. Don't forget the lid.
NEVER VORTEX GENOMIC DNA!
Specimens

You will be divided into groups of 4 people and each group will receive 10 tubes with ethanol preserved adult beetles which will be given a unique identification BMNH number (composed of six digits). **It is extremely important to keep this identifying number with the record of sequence, specimen and any DNA samples from that specimen at all times as it is processed.**

Isolation of DNA

In order to isolate DNA from any specimen, you need first to release the DNA from the cellular compartments in which it is held (nucleus, chloroplast, mitochondria etc) and then to separate it away from other biomolecules present in the extract.

There are a large number of protocols available to isolate DNA both “home-made” and commercial. Each technique has both advantages and disadvantages in terms of speed, complexity, use of toxic chemicals, cost and suitability to particular groups of organisms. Different types of cellular organisation (prokaryote vs. eukaryote, plant vs. animal etc) can require different processing methods and special techniques may be required for particular taxonomic groups.

You will be using a commercial protocol for the isolation of total nucleic acid (genomic and mitochondrial DNA plus RNA); the DNeasy blood and tissue kit from Qiagen. The specimens’ soft tissue is lysed and released from the specimen and then forced through a filter of DNA binding matrix using a centrifuge. The filter is then washed in ethanol-based salt buffers which remove any residual cellular components whilst leaving the nuclear, mitochondrial and ribosomal DNA bound to the matrix. The matrix is then dried and the purified, bound nucleic acids are eluted.

The process involves 4 basic steps:
1. Physical, chemical and enzymatic disruption of the cellular structure.
2. Binding of DNA and RNA to affinity column and removal of other cellular components.
3. Washing the bound nucleic acids.
4. Recovery of nucleic acids from the column (elution).
**Thursday 20th Jan**

1. **Preparation of specimen**

   It is important to use non-destructive sampling wherever possible so great care needs to be taken at this point to keep the specimen as whole as possible – the sample you take for DNA extraction will eventually be recovered and re-joined to the rest of the specimen.

   For larger beetles, (20-30mm) the middle leg including the coxa (plus attached muscle tissue) provide enough tissue from which to extract the DNA. For smaller beetles (5-20mm) use the head and prothorax.

   Each group will get 10 vials, each containing a different species of beetle and representing different families. The tubes are labelled with a six digit BMNH number (British Museum Natural History). You will need to write down the IDs from these labels. These data will be submitted to the Frozen DNA collection database of the NHM and will be later used to print locality labels for the dry voucher specimens that go to the dry collection of the NHM for reference and later morphological investigations.

   1. Make a note of the unique identification numbers (six digits), and any other information from the labels of all your specimens on a separate paper.
   2. Sterilize your forceps by washing them in water (in the 50ml blue-capped tube), wiping them dry with a clean piece of white towel (in order to remove remaining tissue from its tips- use a new piece of towel for each specimen) then dipping into 100% ethanol (in the 50ml blue-capped tube) and air-drying the forceps carefully. Repeat this procedure for each new specimen.
   3. Remove your sample from its tube and place it on a piece of white towel (again, a new piece for each specimen, to avoid cross contamination).
   4. Pull off the right middle leg (ventral view), with the attached muscle tissue. This is most easily done by placing the beetle upside down (ventral side up) and while holding it still with one hand. For smaller beetles, divide the body with a pair of forceps between the pro- and mesothorax. This is most easily done by holding the prothorax with one pair of soft forceps, and the mesothorax with another pair. Gently pull and wiggle.
   5. Place the tissue in a sterilized 1.5ml Eppendorf tube. Label the tube with the BMNH number of the specimen. Leave the tube open to allow the tissue to dry out.
   6. Put the remaining part of the body back into its original vial [make sure that all labels go back inside the tube].
   7. Sterilize your forceps again before turning to the next specimen.

2. **DNA Extraction**

   1. Add 180µl of buffer ATL and 20µl of proteinase K to each tube. Mix by inverting the tubes several times. You are trying to get a balance between even mixing and preservation of the specimen.
   2. Place in the rocking incubator at 56°C over lunch.
   3. Mix by inverting the tubes several times, then spin down quickly in a microcentrifuge to remove any liquid from the lids.
   4. Add 200µl of buffer AL and 200µl of 100% ethanol to each tube (in the 15ml blue capped tubes). Seal and mix IMMEDIATELY by inverting. Spin down.
   5. Unwrap the DNeasy spin column / tube and pipette the mixture from step 4 into the top of the column. **Careful not to touch the membrane with the pipette tip** - you don’t want to scratch it. **Make sure you label the column with the BMNH number.**
   6. Place the column / tube into a microfuge and spin at full speed for 1 minute (ensure that the rotor is balanced before running the microfuge)
   7. Transfer the column to a clean 2 ml tube.
   8. Add 500 µl of Wash Buffer AW1 (not AW2) to the spin column
9. Place the column / tube into a microfuge and spin at full speed for 1 minute (ensure that the rotor is balanced before running the microfuge).
10. Transfer the column to a clean 2 ml tube.
11. Add 500 µl of Wash Buffer AW2 (not AW1) to the spin column.
12. Place the column / tube into a microfuge and spin at full speed for 3 minutes (ensure that the rotor is balanced before running the microfuge).
13. Discard the flow through and return the column to the collection tube.
14. Place the column / tube into a microfuge and spin at full speed for 1 minute (ensure that the rotor is balanced before running the microfuge) - this dry spin ensures that the membrane is completely dry (elution of DNA from the membrane will fail if it retains any of the ethanol-containing wash buffer).
15. Transfer the column to a clean 1.5ml tube. Label the tube with the BMNH number of the specimen you used.
16. Pipette 200 µl of Ultrapure water (in the 15ml blue-capped tube) directly onto the membrane in the spin column (take care to not touch / puncture the membrane with the pipette tip) and leave for 1 minute.
17. Place the column / tube into a microfuge and spin at full speed for 1 minute (ensure that the rotor is balanced before running the microfuge).
18. Put the spin column into a new 1.5ml tube- again, label the tube with the BMNH number, also add “ii” to show it is your second elution. Pipette 200 µl of Ultrapure water directly onto the membrane in the spin column and leave for 1 minute.
19. Place the column / tube into a microfuge and spin at full speed for 1 minute (ensure that the rotor is balanced before running the microfuge).
20. Remove and discard the spin column, and put the caps back on the tubes containing your eluted DNA.

Make sure your DNA is labelled with the BMNH number of the specimen you just extracted!

3. Analysis of purified DNA
The NanoDrop® is a full-spectrum spectrophotometer that measures 1-1.5ul samples. A sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. A pulsed xenon flash lamp provides the light source and a spectrometer is used to analyze the light after passing through the sample. By measuring the absorbance of light at 260nm the nanodrop calculates the concentration of total nucleic acid in your sample. By measuring the absorbance ratios at 260/280 and 260/230 you can also get an idea of the purity of your sample.

You will use the spectrophotometer one group at a time due to limited space. We will then print out your results for you.

4. Voucher specimens
What to do with the specimen after the DNA has been extracted.
Without your extracted specimen saved as a voucher, your DNA sequence will lose value immensely. You might not have an immediate need for your specimen but think ahead and think about other researchers in the future that might want to use your sequence. The researcher might want to determine the species based on the specimen's morphology (if it was not already determined) or want to re-examine the determination because the DNA implies a misidentification. Your specimen might become a type specimen in a descriptive paper if it later turns out to be a new species.
All this means: take care of your specimen during and after extraction.

1. Fill the tubes containing your extracted tissue with water.
2. Pipette off the water and discard.
3. Repeat three or four times to wash off the enzymes and buffers.
4. Fill the tube with 70% ethanol (from the wash bottle) and leave to stand overnight.
5. PCR

In your original groups you will now set up 3 reactions for each sample you processed (10 samples).

You will be amplifying: COI with two primers named SPat and Jerry.
16s with the primers 16Sar and 16Sb2,
28s with the primers 28sFF and 28sDD.

Successful amplification depends on a variety of factors including balancing the amounts of template DNA, primers and other components of the reaction. From previous experience the reaction set-up below will most likely work, but there are no guarantees, this is why 2 elutions are collected. The first elution is likely to be more concentrated, but contain more contaminating impurities that may inhibit the PCR reaction. The 2nd elution will probably contain fewer impurities, but perhaps not enough DNA for the reaction conditions below. For the first experiment, you will use the first elution.

1. You need to make 3 "Master-mixes" – one for each gene you are amplifying. Label 3 x 1.5ml tubes as “CO1”, “16S” or “28S”. Add the reagents in the table below to each tube on ice. This will make enough mix for all 10 samples plus a negative control.

Don’t forget to vortex and spin down each tube before use. Do not use anything that has not been completely thawed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per sample</th>
<th>Volume for 10 samples and a negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd H2O (sterile)</td>
<td>37.6 µl</td>
<td>432.4 µl</td>
</tr>
<tr>
<td>Buffer</td>
<td>5 µl</td>
<td>57.5 µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>2 µl</td>
<td>23 µl</td>
</tr>
<tr>
<td>Primer F</td>
<td>1.2 µl</td>
<td>13.8 µl</td>
</tr>
<tr>
<td>Primer R</td>
<td>1.2 µl</td>
<td>13.8 µl</td>
</tr>
<tr>
<td>*dNTPMix</td>
<td>2 µl</td>
<td>23 µl</td>
</tr>
<tr>
<td>Taq</td>
<td>0.1 µl</td>
<td>1.2 µl</td>
</tr>
</tbody>
</table>

2. After all ingredients have been added to the tube, vortex so that the Taq is evenly dispersed throughout the mastermix.
3. Label your PCR tubes so you know which sample and which mastermix each contains.
4. Transfer 49µl of master-mix to each tube.
5. Add 1µl* of the DNA template to each PCR tube. Add 1µl of sterile water to your negative control.

*1µl is a standard volume for the first time you do the experiment, however the amount of DNA can vary, sometimes it might need to be diluted, sometimes you might need to take more than 1µl. If changed make sure the ddH2O is changed accordingly to make a total volume of 50µl.

NOTES

- Primer concentrations should normally be between 0.1-1uM in a PCR reaction.
- You will use a ready-mix of the four dNTP’s. dNTPs are added at 2.5mM of EACH of the four dNTP’s. This is the standard concentration suggested in most PCR protocols.
- Taq enzyme comes in stock tubes with a concentration of 5units/ul (1 unit defined as the amount of enzyme that incorporates 10nmoles of dNTP’s into acid-insoluble form in 30 minutes at 72°C). Manufacturers always grossly over-suggest the amount of Taq to use for each reaction. More Taq can increase PCR yield but can also reduce specificity. The Taq enzyme is the most expensive component of the PCR-reaction. The amount of Taq in this reaction has been optimised.
- The optimal concentration of MgCl2 has already been determined empirically for this reaction, the MgCl2 must be determined for every primer pair.
6. Place your rack of tubes into the ice bucket at the front of the class. When everyone is ready, we will load them into a thermocycler PCR machine, programmed as follows:

<table>
<thead>
<tr>
<th>16S and 28S</th>
<th>CO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>94ºc 5mins</td>
<td>x1</td>
</tr>
<tr>
<td>94ºc 30secs</td>
<td>x30</td>
</tr>
<tr>
<td>50ºc 30secs</td>
<td></td>
</tr>
<tr>
<td>72ºc 30secs</td>
<td></td>
</tr>
<tr>
<td>72ºc 7mins</td>
<td>x1</td>
</tr>
<tr>
<td>10ºc hold</td>
<td>x1</td>
</tr>
</tbody>
</table>

6. **Gel Electrophoresis**

Before you use a PCR product to obtain sequence data you must first check that you have produced a product from your specimen DNA extraction and assess that the product is of the expected size and is therefore likely to be the correct product. You also need to check the purity as there may be more than one product, or the DNA may be degraded. Also the concentration needs to be assessed to ensure that there is sufficient for the proposed protocols that will follow. This is initially done by running out a sample of your PCR product on an agarose gel together with a reference DNA ladder containing DNA fragments of known size and quantity. Cross referencing your PCR product to the bands on the ladder enables the size, quality and yield to be assessed.

Preparation of an agarose gel is as follows –

1. Assemble gel casting rig with combs (this will accommodate all the samples processed as well as reference size markers).
2. Make sure it’s level.
3. Measure out the TAE (Tris – Acetate – EDTA)- Your tanks are labelled with the appropriate volume.
4. Add Agarose to TAE- again see your tank for the amount.
5. Microwave on medium power until gently boiling, swirling often to avoid superheating. Liquid should be transparent with no particulates.
6. Cool to 70 ºc under cold tap, swirling to avoid solidification. 70 ºc is hand-hot.
7. Pour liquid into gel casting rig.
8. Leave for 30min to harden. It will look opaque.
9. Remove the combs gently.
10. Put gel in the Electrophoresis tank. Make sure the TAE-buffer covers the Gel completely, and that the Gel is in the middle.
11. Add 1µl of loading buffer Dye to 33 wells of a V-bottom 96 well plate.
12. Add 3µl of your PCR product with the 1ul of loading buffer Dye in the 96 well plate, pipette several times to mix.
13. Add the 4 µl to a well – note the order in which you load your samples.
14. Load 1ul of the reference ladder to a well.
15. Attach the lid. Start the voltage on approx 100V depending on how detailed separation you need (lower voltage, slower to run but more detailed separation).
16. Leave to run for ca. 20-30min [depending on the voltage]. The two blue loading dye bands should have a distinct gap in between.
17. The samples should not run out over the edge of the gel!
18. Turn off the Voltage, lift up the Gel rig out of the tank and take the gel to the UV photo-room for visualisation of the DNA.
19. Print a picture.
20. Dispose of the gel in the yellow sack.
21. The PCR reactions that correspond to a product band on the gel can now be progressed. (Where no product is seen the reaction has failed and the sample will need to be repeated, perhaps with more/less DNA template).

**Remember your voucher specimens!**

Carefully transfer the legs or heads of your extractions into the original tubes, with the rest of the specimen- making sure all the labels are correct.