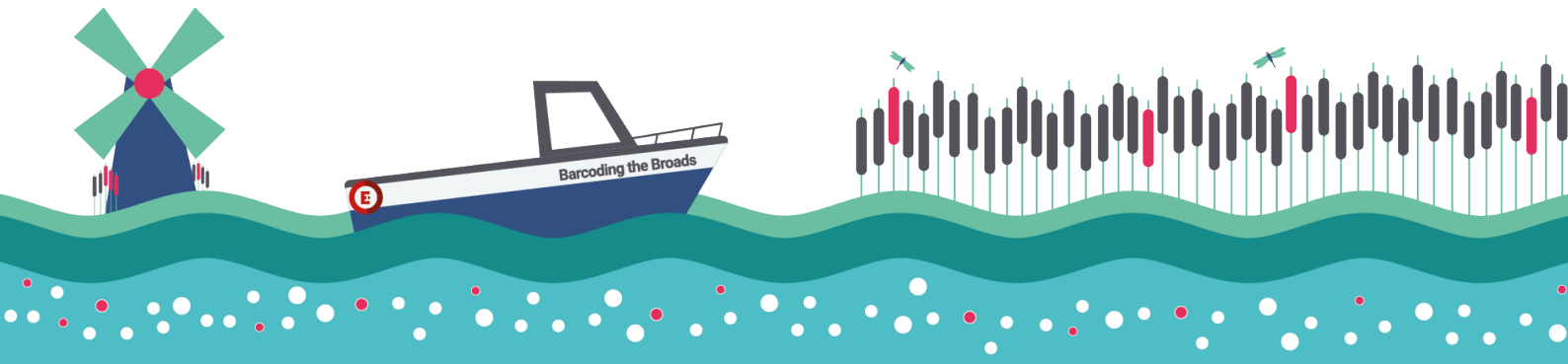
Welcome to Barcoding the Broads

A Wellcome-funded programme of public engagement events and activities to explore biodiversity on the Norfolk Broads, led by the Earlham Institute as part of our work on the Darwin Tree of Life project.

Content:



DNA Barcoding Protocol - Plants

All Equipment, Consumables, and Reagents

Equipment:	Check
-20 °C freezer	
Balance	
BlueGel electrophoresis system	
Centrifuge	
Computer/Laptop with internet connection	
Conical flask	
Eppendorf and PCR tube rack	
Ice bucket	
Lab book	
Media bottle	
Metal forceps	
Metal spatula	
Microwave	
MiniPCR thermal cycler	
Nitrile gloves	
P10, P20, P200 and P1000 pipettes	
Plastic pestles	
Scissors	
Waste bin	
Water bath (or mug and kettle)	

Consumables:	Check
0.2 mL PCR tubes	
1.5 mL eppendorf tubes	
10 mL plastic syringe	
10 µL, 20 µL, 200 µL and 1000 µL pipette tips	
Aluminium foil	
Ice	
Marker pen	
Parafilm M	
Plastic weigh boats	
Sanger sequencing labels (Genewiz)	
Sterile/Distilled water	

Reagents:	Check
1 x TBE buffer [made through a ten-fold dilution of 10 x TBE buffer in sterile/ distilled water]	
10 % chelex solution [made by mixing 1 g chelex into 9 mL sterile/distilled water]	
100bp DNA ladder	
Agarose	
DNA loading dye	
rbcL primers (forward and reverse, Eurofins)	
Ready-to-Go PCR beads	
SYBR Safe DNA gel stain	

DNA Barcoding Protocol - Plants

STAGE 1] Sample Collection and DNA Extraction

Equipment:	Check
Balance	
Centrifuge	
Eppendorf and PCR tube rack	
Lab book	
Media bottle	
Metal forceps	
Metal spatula	
Nitrile gloves	
P200 pipette	
Plastic pestle	
Scissors	
Waste bin	
Water bath (or mug and kettle)	

Consumables:	Check
1.5 mL eppendorf tubes	
10 mL plastic syringe	
200 µL pipette tips	
Aluminium foil	
Marker pen	
Parafilm M	
Plastic weigh boats	
Sterile/Distilled water	

Reagents:	Check
10 % chelex solution [made by mixing 1 g chelex with 9 mL sterile/distilled water]	

Notes

DNA Barcoding Protocol - Plants

STAGE 1] Sample Collection

The first task is to collect and document a plant sample, ensuring you respect the study environment and have permission to collect in the chosen location. Only a small amount of material is needed - typically a single leaf. This can be removed by hand, or with a scalpel or scissors, and then stored in a labelled vial.

Metadata

As much information as possible about the sample should be recorded alongside the collection.

This includes:

- Photos of the plant
- Latitude, longitude, temperature and elevation of the study location
- Time and date of collection
- Description of the plant and the habitat it grows in
- Sample number and name of the collector

All sample information should be logged in a lab book or on a computer.

An example metadata collection table can be found on page 9

DNA Extraction

Wear gloves throughout to avoid contamination

Use a fresh tip every time you pipette to avoid contamination

The DNA, found in the cell nucleus, is extracted from the leaf and separated from contaminants using simple physical and chemical methods:

1. Add 100 µL of 10 % chelex solution to a 1.5 mL eppendorf tube and use a marker pen to label the top and side of the tube with the sample name/number
2. With the leaf rested on a plastic tray, remove a small half-circle of the sample - roughly the size of a grain of rice - with the reverse (wide) end of a 200 µL pipette tip
3. Transfer the piece of leaf to the eppendorf tube and crush thoroughly into the chelex solution using a plastic pestle until it is completely broken apart
4. Close the eppendorf tube and seal it tightly with parafilm. Heat the contents for 10-15 mins in a water bath (85-90 °C) or in a mug containing boiling water. Avoid contamination by ensuring the lid of the tube isn't submerged
5. After heating, remove the eppendorf tube from the water and use a centrifuge (e.g. 6000 rpm for 1 min) to pellet the solid material. Alternatively, allow the contents to settle for 15 mins until two distinct layers form. The DNA is found in the top layer of liquid that forms above the solid material, 2 µL of this is used for the polymerase chain reaction (PCR). Handle the tube carefully from this stage to avoid disturbing the solids at the bottom

DNA Barcoding Protocol - Plants

STAGE 2] Polymerase Chain Reaction (PCR)

Equipment:	Check
-20 °C freezer	
Computer/Laptop for PCR software	
Eppendorf and PCR tube rack	
Ice bucket	
Lab book	
MiniPCR thermal cycler	
Nitrile gloves	
P20 pipette	
Waste bin	

Consumables:	Check
0.2 mL PCR tubes	
20 µL pipette tips	
Ice	
Marker pen	
Plastic weigh boats	
Sterile/Distilled water	

Reagents:	Check
Ready-to-Go PCR beads	
rbcL primers (forward and reverse, Eurofins)	

Notes

DNA Barcoding Protocol - Plants

Wear gloves throughout to avoid contamination

Use a fresh tip every time you pipette to avoid contamination

STAGE 2] Polymerase Chain Reaction (PCR)

PCR is used to amplify (make millions and billions of copies of) a specific region of DNA.
For plants the DNA barcode region is **rbcL**.

1. Transfer a ready-to-go PCR bead (containing Taq polymerase, nucleotides, stabilisers and reaction buffer) to a 0.2 mL PCR tube and use a marker pen to label the top and side of the tube with the sample name/number
2. Add 20 µL sterile/distilled water to the PCR tube and allow the bead to completely dissolve
3. Add 1.5 µL of the forward primer and 1.5 µL of the reverse primer to the PCR tube
4. Add 2 µL of extracted DNA (taken from the top layer of liquid that forms above the solid material) to the PCR tube. Mix thoroughly with a pipette tip before firmly closing the lid. The final volume of the solution in the PCR tube is 25 µL. A negative control (used to confirm the experimental results are valid) is prepared by repeating the steps above but with 2 µL sterile/distilled water replacing the 2 µL of extracted DNA
5. Transfer the PCR tubes to the miniPCR machine. Screw the lid of the machine closed and then start the thermal cycling protocol using the miniPCR software

Thermal Cycling

Amplification of the DNA barcode region happens in three steps:

1. *Denaturation*, where the double-stranded DNA is heated to separate the strands
2. *Annealing*, where the primers mark/flank the DNA barcode region by binding to the separated strands of DNA
3. *Extension*, where the *Taq* polymerase synthesises new DNA strands.

These steps are repeated (cycled) to exponentially produce exact copies of the DNA barcode region.

Thermal cycling programme for rbcL (1 hr 23 min duration, with heated lid) is:

Initial denaturation = 94 °C for 60 sec

| *Denaturation* = 94 °C for 15 sec
| *Annealing* = 54 °C for 15 sec
| *Extension* = 72 °C for 30 sec
| Number of cycles = 35

Final extension = 72 °C for 300 sec

DNA Barcoding Protocol - Plants

STAGE 3] Gel Electrophoresis and Sanger Sequencing

Equipment:	Check
-20 °C freezer	
P10, P20, P200 and P1000 pipettes	
Balance	
BlueGel electrophoresis system	
Computer/Laptop with internet connection	
Conical flask	
Eppendorf and PCR tube rack	
Ice bucket	
Lab book	
Metal spatula	
Microwave	
Nitrile gloves	
Waste bin	

Consumables:	Check
0.2 mL PCR tubes	
1.5 mL eppendorf tubes	
10 mL plastic syringe	
10 µL, 20 µL, 200 µL and 1000 µL pipette tips	
Ice	
Marker pen	
Parafilm M	
Plastic weigh boats	
Sanger sequencing labels (Genewiz)	
Sterile/Distilled water	

Reagents:	Check
1 x TBE buffer [made through a ten-fold dilution of 10 x TBE buffer in sterile/distilled water]	
Agarose	
100bp DNA ladder	
DNA loading dye	
SYBR Safe DNA gel stain	

Notes

DNA Barcoding Protocol - Plants

STAGE 3] Gel Electrophoresis and Sanger Sequencing

Gel/Sample Preparation

Use a fresh tip every time you pipette to avoid contamination

Wear gloves throughout to avoid contamination

Gel electrophoresis confirms whether the polymerase chain reaction (PCR) was successful. The technique mobilises DNA by charge and DNA fragments are separated by size, with smaller fragments moving further through the gel.

1. Weigh out 0.4 g agarose and mix with 20 mL of 1 x Tris/Borate/EDTA (TBE) buffer in a conical flask
2. Microwave the mixture for 20-30 seconds or until the agarose has completely dissolved, ensuring the solution does not boil over. The solution should look completely transparent. Once the solution has cooled slightly, add 2 µL of red SYBR Safe DNA gel stain (NB: this reagent is light sensitive so keep it away from light when not in use)
3. Gently swirl the solution and pour into the gel tray with a comb insert, giving 9 large wells (20 µL capacity) or 13 small wells (10 µL capacity). Pour the solution slowly to avoid bubbles and then allow the gel to set for approximately 20 minutes
4. Once the gel has set, carefully remove the comb and transfer the gel tray into the main sample tray. Fill the main sample tray with 35 mL of 1 x TBE buffer to cover the gel. DNA samples can now be prepared for analysis on the gel
5. Add 5 µL of amplified DNA sample (post-PCR) to 1 µL loading dye in a 0.2 mL PCR tube and mix with a pipette tip. Repeat with the negative control sample. The DNA ladder (used to determine the approximate size of DNA samples on the gel) is similarly prepared by combining 5 µL 100 bp DNA ladder with 1 µL loading dye in a 0.2 mL PCR tube
6. Carefully add the 6 µL DNA/dye samples to the wells on the gel (one sample per well) then seal the tank with the orange lid and press the power button. Progress of the gel is monitored by watching the dye move through the gel or by turning on the blue light

Sanger Sequencing

DNA (Sanger) sequencing is used to determine the order of the A, C, T & G building blocks in the DNA barcode region. If gel electrophoresis confirms the PCR experiment was successful then 20 µL of the amplified DNA sample can be transferred to a 1.5 mL Eppendorf tube and logged for collection/analysis via the Genewiz website.

The eppendorf tube is sealed with parafilm, a Sanger sequencing label is attached and then the sample is dropped off for collection (e.g. in the John Innes Centre reception before 2:30 pm). Sanger sequencing progress is tracked via the Genewiz website and **.ab1** files for the DNA Subway can be downloaded once the process is complete.

DNA Barcoding Protocol - Plants

STAGE 4] DNA Subway

Equipment

Computer/Laptop with internet connection

Software

DNA sequences are analysed with the DNA Subway (https://dnasubway.cyverse.org). The platform is accessed with a CyVerse account or as a Guest (for the latter, projects can't be saved long-term). The Blue Line is selected to start a new project, "Barcoding: rbcL" is chosen as the project type and .ab1 files from the Genewiz website (one forward and one reverse per sample) are uploaded. A project title and short description can be added here too.

Step 1: Assemble Sequences

First, DNA sequences are viewed and automatically trimmed before the forward and reverse sequences for each sample are paired up. A consensus sequence is built from the forward/reverse pairs which can then be manually trimmed at both ends, ideally keeping the final sequence length above 400 bp.

Step 2: Add Sequences

Second, the consensus sequence is compared with online databases using a tool called BLAST (Basic Local Alignment Search Tool) to identify the plant from its DNA barcode region. Suggested species names and images are shown alongside an analysis of how close the consensus sequence matches those in the online databases. If the database sequences show <95% similarity to the consensus sequence then this could indicate the discovery of a new species. A range of rbcL reference data sets (e.g. common plants) can then be added to compare against the consensus sequence in the next step.

Step 3: Analyse Sequences

Third, multiple sequences (the consensus sequence and the reference data sets) are compared using a tool called MUSCLE (MUltiple Sequence Comparison by Log Expectation). Grey areas indicate a match with the overall consensus whereas coloured bars/letters show where individual sequences differ. After automatically trimming the multiple sequence alignments, phylogenetic trees can be made to visualise the relationship between plant species. Two types of phylogenetic tree can be made: neighbour joining (NJ) and maximum likelihood (ML). For both, an outgroup (i.e. the plant species in the data set that is most dissimilar to the one being studied) is chosen to act as a reference point for determining where the root and branches of the tree are placed.

⇢ DNA sequences of high enough quality can be exported to GenBank

Barcoding the Broads Metadata Collection table

Name:_____ **Date (dd/mm/yy):**_____ **Sample (plant/insect/fungus):** _____

Organisation/School:_____ **Collection Location:**_____

Specimen Collection Data								
Sample Number	**Photo taken**	**Time**	**Latitude**	**Longitude**	**Temp (˚C)**	**Predicted Species**	**Habitat Description**	**Notes**
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	☐							
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