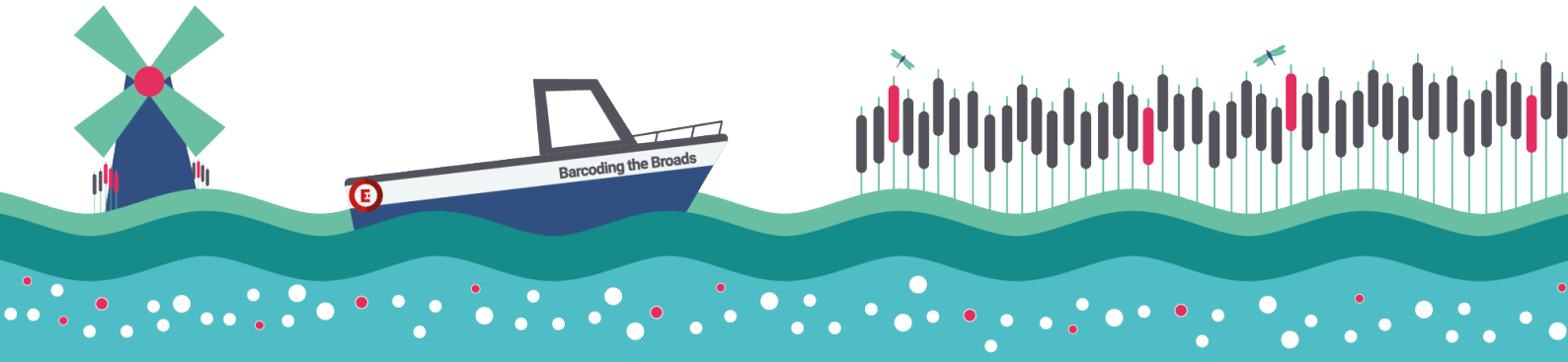
Welcome to Barcoding the Broads

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 - Invertebrates 

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	
10 µL, 20 µL, 200 µL and 1000 µL 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/Azenta)	
Sterile/Distilled water	

Reagents:	Check
1 x TBE buffer [NB: if using **10 x TBE** buffer, dilute ten-fold in sterile/distilled water]	
10% chelex solution [made by mixing 1g chelex into 9 mL sterile/distilled water]	
100bp DNA ladder	
Agarose	
DNA loading dye	
Ethanol (for cleaning)	
CO1 primers (forward and reverse)	
Ready-to-Go PCR beads	
SYBR Safe DNA gel stain	

DNA Barcoding Protocol - Invertebrates - 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	
200 µL 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 1g chelex into 9mL sterile/distilled water]	
Ethanol (for cleaning)	

Notes

DNA Barcoding Protocol - Invertebrates - STAGE 1

Sample Collection and DNA Extraction

The first task is to collect and document an invertebrate sample, ensuring you respect the study environment and have permission to collect in the chosen location. Only a small amount of material is needed for DNA barcoding.

Metadata

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

This includes:
- Photos of the invertebrate
- Latitude, longitude, temperature and elevation of the study location
- Time and date of collection
- Description of the invertebrate and the habitat it was found
- 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

DNA is extracted from the invertebrate and separated from contaminants using 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 Carefully remove a small section of your invertebrate sample, wearing gloves to avoid contamination - e.g. smaller specimens like ants and spiders can be halved or used whole, or a few millimeters of leg/antenna can be taken from larger specimens like beetles and butterflies

3 Transfer the section of invertebrate sample to the labelled 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 Use a centrifuge (e.g. 10000 rpm for 2 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. Handle the tube carefully to avoid disturbing the solid layer at the bottom

DNA Barcoding Protocol - Invertebrates - STAGE 2

Polymerase Chain Reaction (PCR)

Equipment:	Check
-20 °C freezer	
Computer/Laptop for miniPCR software	
Eppendorf and PCR tube rack	
Ice bucket	
Lab book	
MiniPCR thermal cycler	
Nitrile gloves	
20 µL and 200 µL pipettes	
Waste bin	

Consumables:	Check
0.2 mL PCR tubes	
20 µL and 200 µL pipette tips	
Ice	
Marker pen	
Plastic weigh boats	
Sterile/Distilled water	

Reagents:	Check
Ready-to-Go PCR beads	
CO1 primers (forward and reverse)	
Ethanol (for cleaning)	

Notes

DNA Barcoding Protocol - Invertebrates - STAGE 2

Polymerase Chain Reaction (PCR)

Wear gloves throughout to avoid contamination

Use a fresh tip every time you pipette to avoid contamination

PCR is used to amplify (make billions of copies of) a specific region of DNA. For invertebrates, the DNA barcode region is called **CO1**.

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. Primer sequences are:

   CO1-M13-forward: 5'-TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG-3'
   CO1-M13-reverse: 5'-CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA-3'

4. Add 2 µL of extracted DNA (from the top layer of liquid that forms above the solid material at the end of STAGE 1) 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 (without DNA) is prepared by repeating the steps above using 2 µL sterile/distilled water instead of 2 µL 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 CO1 (1 hr 57 min duration, with heated lid) is:

Initial denaturation = 94 °C for 60 sec

| *Denaturation* = 95 °C for 30 sec
| *Annealing* = 50 °C for 30 sec
| *Extension* = 72 °C for 45 sec
| Number of cycles = 35

Final extension = 72 °C for 180 sec

DNA Barcoding Protocol - Invertebrates - STAGE 3

Gel Electrophoresis and Sanger Sequencing

Equipment:	Check
-20 °C freezer	
10 µL, 20 µL, 200 µL and 1000 µL 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/Azenta)	
Sterile/Distilled water	

Reagents:	Check
1 x TBE buffer [NB: if using **10 x TBE buffer**, dilute ten-fold in sterile/distilled water]	
100bp DNA ladder	
Agarose	
DNA loading dye	
Ethanol (for cleaning)	
SYBR Safe DNA gel stain	

Notes

DNA Barcoding Protocol - Invertebrates - STAGE 3

Gel Electrophoresis

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. Mix 0.4 g agarose with 20 mL of 1 x Tris/Borate/EDTA (TBE) buffer in a conical flask. NB: if using **10 x TBE buffer**, remember to dilute ten-fold in sterile/distilled water

2. Microwave the mixture for 20-30 seconds or until the agarose has completely dissolved, ensuring the solution does not boil over. Once the solution has cooled slightly, add 2 µL of SYBR Safe DNA gel stain (NB: reagent is light sensitive so store in the dark)

3. While it's still warm, gently swirl the solution and carefully pour into the gel tray with a comb insert, giving 9 large wells (20 µL capacity) or 13 small wells (10 µL capacity). Allow the gel to set completely (approximately 15 minutes)

4. Once the gel has set, carefully remove the comb and transfer the gel tray into the main sample tray. Cover the gel with 35 mL of 1 x TBE buffer (NB: if using **10 x TBE buffer**, remember to dilute ten-fold in sterile/distilled water). 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 of 100bp DNA ladder with 1 µL loading dye in a 0.2 mL PCR tube

6. Carefully add the 6 µL of each DNA/dye sample to the wells on the gel (one sample per well) then seal the tank with the lid and press the power button. Progress of the gel is monitored by turning on the blue light (viewed through the hole in the dark box)

Sanger Sequencing

DNA (Sanger) sequencing is used to determine the order of 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/Azenta website.

Progress with sample analysis is tracked via the Genewiz/Azenta website and **.ab1** files for the DNA Subway can be downloaded once the process is complete. Speak to your local Genewiz/Azenta account manager for more information about submitting samples for DNA sequencing and reviewing the results.

DNA Barcoding Protocol - Invertebrates - STAGE 4

DNA Subway

Equipment

Computer/Laptop with internet connection

Software

DNA sequences are analysed using the "Blue Line" on 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). Select "Barcoding: CO1" as the project type and upload **.ab1** files from the Genewiz/Azenta website (one forward and one reverse per sample). A project title and short description can be added here too.

Step 1: Assemble Sequences

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

Step 2: Add Sequences

The consensus sequence can be compared with online databases using a tool called BLAST (Basic Local Alignment Search Tool) to identify invertebrate species from the CO1 DNA barcode region. Suggested species names are shown alongside an analysis of how close the consensus sequence matches those in the online databases. A range of CO1 reference data sets (e.g. common insects) can be added to compare against the consensus sequence in the next step.

Step 3: Analyse Sequences

Multiple sequences can be 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. The multiple sequence alignments can be trimmed and then phylogenetic trees can be made to visualise the relationship between invertebrate species. Two types of phylogenetic tree are available: neighbour joining (NJ) and maximum likelihood (ML). For both, an outgroup (i.e. the invertebrate 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.

⇢ More information about the DNA Subway can be found under the "DNA Sequencing and Analysis" tab on the Barcoding the Broads webpage. Please log your DNA barcoding results on the Darwin Tree of Life community map/database to support sampling for full genome analysis and request assistance with species identification.

Barcoding the Broads Metadata Collection Table

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