

Appendix 6

Methods and protocols

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1. DNA Extraction

Note ALL WATER IS PURITE DEIONIZED WATER UNLESS STATED AS “STERILE” OR “ULTRA” WHICH IS COMMERCIALY SUPPLIED DNASE AND RNASE FREE

For Pure Bacteria (cultures)

On agar media

Scrape a few colonies into 35ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99°C – proceed to PCR protocol A

In Liquid media

Add 3ul of an overnight culture to 32ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99°C – proceed to PCR protocol A

Plasmid DNA from Clones

Add 3ul of an overnight culture to 21.45ul sterile ultra water and boil for 20 minutes in gradient PCR machine at 99°C – proceed to PCR protocol B

For Environmental (wood) samples

Take 500mg of sample, place in 1.5ml microfuge tube and freeze dry at –80°C for one week) Grind environmental sample using liquid nitrogen and a sterile mortar and pestle. Proceed with CTAB lysis procedure or Qiagen DNAeasy Plant Mini kit

DNA Extraction using CTAB lysis Buffer: used for BACPOLES project until August 2004

Suspend 0.015-0.025g of material in 500 μ l 75°C CTAB lysis buffer (2%CTAB, 100mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1% PVPP) and vortex for 30 seconds

Incubate Tubes @75°C in water bath for 15 Min, vortexing every 5 min for 30 seconds

Allow to cool and add 0.5volume (250 μ l) Chloroform:Isoamyl alcohol (24:1)

Vortex for 30 seconds

Centrifuge @10,000rpm for 10 min

Collect supernatant and put in fresh tube

Centrifuge again for 10 min @10,000rpm

Collect supernatant and put in fresh tube

Extract supernatant mix with 1 volume (500 μ l) Chloroform:Isoamyl alcohol (24:1)

Centrifuge again for 10 min @10,000rpm

Collect supernatant and put in fresh tube

Centrifuge again for 10 min @10,000rpm and put in fresh tube

Add 0.5 volume (250 μ l) 30% PEG (Polyethylene glycol) 8000

Incubate on ice for 3 hours or leave overnight in –20°C freezer

Pellet by centrifuge in a chilled centrifuge 4-8°C @10,000rpm for 15 minutes

Pour off PEG and allow pellet to dry very well about 20 mins

Ethanol Precipitation Step

Resuspend pellet in 500 μ l 70% Ethanol (10mM Sodium Acetate)

Centrifuge @10,000rpm for 20 minutes

Remove ethanol and dry pellet

Resuspend pellet in 100-150ml TE Buffer (10mM Tris-HCl, 1mM EDTA pH 8.0)/Sterile Water

DNA Extraction using QIAGEN DNeasy Plant Mini Kit: used for BACPOLES project from August 2004 to project completion

Full protocol given on manufacturers web page (www1.qiagen.com)

DNA Cleanup

Repeat last 4 steps in CTAB protocol (Treat 150 μ l of DNA suspension as dry pellet) if using CTAB protocol

Use the QIAquick Nucleotide Removal Kit from QIAGEN. Full protocol given on manufacturers web page (www1.qiagen.com).

2. Polymerase Chain Reaction (PCR)

Note: EVERYTHING MUST BE DEFROSTED ON ICE AND KEPT ON ICE WHILST DISPENSING

PCR Profile

Denaturing..... 1Cycle 94°C 5 Min

*Annealing.....*35 Cycles 94°C 1 Min followed by 51-58°C depending on primer set (see annealing temperatures in Appendix 2) 1 Min and 72°C 1 Min

*Extension.....*1 Cycle 72°C 10 Min

PCR Master Mix for 30 samples

Protocol A – To be used for bacterial cultures and environmental samples

150 μ l 10X Buffer

30 μ l MgCl₂ (25mM) - Promega/ 15 μ l MgCl₂ (50mM)-Invitrogen

30 μ l dNTPs (10mM)

45 μ l Forward Primer

45 μ l Reverse Primer

1050 μ l Sterile Water if using Promega bead/1065 μ l Sterile water if using invitrogen Taq

Dispense 45 μ l of mix per tube. Add add a promega bead or 0.15 μ l Invitrogen Taq polymerase plus 4.7 μ l template DNA to each tube. Centrifuge reaction tubes at max speed for 30 seconds to ensure that all components are mixed

Protocol B – To be used for Clones (Plasmid DNA)

90 μ l 10X Buffer

54 μ l MgCl₂ (50mM)

15 μ l dNTPs (10mM)

15 μ l Forward Primer (20uM M13F)

15 μ l Reverse Primer (20uM M13R)

4.5 μ l Taq polymerase

Add 5.55 μ l of mix to tubes containing boiled samples. Centrifuge reaction tubes at max speed for 30 seconds to ensure that all components are mixed.

Cleanup of PCR Products

Using QIAGEN QIAquick PCR Purification kit. Full instructions given on manufacturers web site (www1.qiagen.com).

Agarose Gel Electrophoresis

PCR products are separated electrophoretically and visualized by UV light using Ethidium Bromide (EtBr) as a fluorescent label. EtBr added in concentration of 5 μ g/ml.

For looking at all DNA types:-

For long fragments (eg. 8F-1492R 1400bp products) can use two combs per gel and run for approx 1 hour at 100V

For shorter fragments (eg. UNV2-UNV3 220 bp products) must run 1 comb per gel run at 100V for 1.5-2 hrs.

1.5% = 1.5g Agarose in 100ml 1XTAE buffer – melt on high in microwave for 2 mins and then add 1.5 μ l EtBr

For looking at RFLPs:-

3% = 3g Agarose in 100ml 1XTAE buffer– melt on high in microwave for 3.5 mins and then add 1.5 μ l EtBr

Gels take approximately 20mins to set

Note: Always make sure buffer in tanks is same as buffer in gels. When running products for gel extraction, always use fresh buffer. Always run gels from black electrode to red electrode, keep lids on tanks at all time to avoid electrocution.

Cleanup of PCR Product From Agarose Gel

Using QIAGEN QIAEXII Agarose Gel Extraction Kit. Full instructions given on manufacturers web site (www1.qiagen.com).

3. Denaturing Gradient Gel Electrophoresis (DGGE)

A nested PCR is performed to obtain PCR products of the appropriate length for DGGE.

Extracted DNA initially amplified with 8F-1492R primers to obtain sequences of 1400bp.

These products in turn amplified with UNV2 (GC clamp)-UNV3 primers to produce smaller (approx 220bp) products

PCR products are separated using a 30% and 70% urea-formamide denaturing gradient within a polyacrylamide gel.

Stock Solutions – will last no more than 1 week at 4^oC

0% Denaturant

16.2ml 40% Acrylamide:Bis-Acrylamide (37.5:1)

2ml 50X TAE buffer

81.8ml ddH₂O

adjust to 100ml with ddH₂O if necessary store in a darkened container in the fridge @4^oC

100% Denaturant

16.2ml 40% Acrylamide:Bis-Acrylamide (37.5:1)

2ml 50X TAE buffer

42g Urea

40ml Formamide

adjust to 100ml with ddH₂O if necessary, warm to dissolve urea. Store in a darkened

container in the fridge @4^oC and warm at no more than 40^oC in a waterbath to dissolve urea before use, bringing back to room temp before each run.

Note: never dissolve urea in >40 ^oC as gel will not set

20% Ammonium Persulfate (APS)

0.2g Ammonium persulfate

1ml ddH₂O

(prepare fresh before each run)

Preparation of Gradient:

30% Solution

17.5ml 0% stock

7.5ml 100% Stock

add 126 μ l 20% Ammonium persulfate and 12.6 μ l TEMED before pouring

70% Solution

17.5ml 100% stock

7.5ml 0% Stock

add 126 μ l 20% Ammonium persulfate and 12.6 μ l TEMED before pouring

also add 50 μ l gradient dye to this solution to aid visualisation of mixing of gradients

Note: make sure that you add the Ammonium persulfate and TEMED while the magnetic stirrers are going as these are the reagents which help the gel set so they must be mixed right through the solution

0% Solution

Prepare 5ml of 0% solution to top up the top of the gel where the well comb is inserted (add 25.2 μ l 20% Ammonium persulfate and 2.52 μ l TEMED keep shaking with your hand and pipette on to top of gel using 1ml pipette)

Add 50 μ l gradient dye to this solution to aid visualisation of wells when loading samples

Running and visualisation of gels

Gels are run at 60°Celsius, 100V for 16hrs.

Gels are visualized using UVr and EtBr staining

Stain for 30 mins in 30 μ l EtBr:300ml dh₂O

Destain for 15 mins (no longer) in 300 ml dh₂O

Excise bands of interest and extract DNA

Recovery of DNA from Polyacrylamide Gels

Using the QIAEX II Gel Extraction Kit from QIAGEN (www1.qiagen.com), following the manufacturers protocol..

Post DGGE PCR and Cleanup

Recovered DNA is re-amplified using the same primer set (UNV2-UNV3) but without the GC clamp and subjected to the QIAGEN PCR purification kit.

4. Cloning

Using the Invitrogen Kit: TOPO TA Cloning (www.invitrogen.com)

Ligation

It is best to make up a mastermix of the ligation reaction mixture and dispense 1.67 μ l into each reaction tube.

1.00 μ l Sterile water

0.33 μ l Salt Solution

0.33 μ l TOPO Vector

Add the 0.33 μ l PCR product to be inserted (from a standard PCR reaction (Protocol A above).

Gently mix by tapping the tube (*do not* use a pipettor!) and incubate at room temperature for 5 minutes.

After incubation, place the reaction on ice (or store overnight at -20°C).

Transformation into Competent Cells:

Add 16 µl of the One Shot Chemically Competent *E. coli* cells to each cloning reaction.

Note: competent cells must be stored at -80 °C, defrosted and dispensed on ice and cannot be refrozen

Incubate on ice for 5 to 30 minutes.

Heat-shock the cells for 30 seconds at 42°C without shaking.

Immediately transfer the tubes to ice.

Add 83 µl of the room-temperature SOC medium.

Incubate tubes horizontally for 1 hour in the 37° shaking incubator at 200 rpm.

Using sterile technique, spread 40 µl of IPTG then 40 µl of XGAL onto each prewarmed plate

Using sterile technique, spread 50 µl (do two plates) from each transformation onto a prewarmed LB plate. Let stand right-side up for about 5 minutes to allow the cells to adhere to the agar, then invert and incubate overnight at 37°C.

Note: do not incubate for longer as get an over abundance of false positives as the ampicillin is used up.

Screening of Colonies

Using sterile technique, pick 10 white colonies off the agar plates and drop into 10 microcentrifuge tubes each containing 1ml LB (with 100mg/ml ampicillin).

Note: no colony should grow unless it has an insert. The *ccdB* lethal gene in the plasmid should be disrupted if the insert is in place thus allowing the clones to grow in an ampicillin rich environment. However random mutations do sometimes occur allowing growth of other colonies. Colonies are further analysed using PCR.

Incubate colonies overnight @ 37°C on a 200rpm shaker table.

Add 3µl of overnight culture to 20µl sterile water in a PCR tube, quick centrifuge and boil on Gradient PCR machine block for 20 mins.

Perform specific PCR reaction for clones (Protocol B above):

3µl 10X Buffer

1.8µl 25mM MgCl₂ **or** 0.9 ml 50mM MgCl₂

0.5µl 10uM dNTPs

0.5 µl 20uM primer M13F

0.5 µl 20uM primer M13R

1 unit Taq polymerase (0.7µl)

PCR profile recommended:

Denaturing 1 cycle 94°C for 10 min

Annealing 30 cycles 94°C for 1 min, followed by 55°C for 1 min and 72°C for 1 min

Extension 1 cycle 72 °C for 10 min

Hold @ 4°C

visualise using agarose gel electrophoresis (1.5% gel). Successful clones should be approx 450 - 600bp in size whereas false positives will yield products of different sizes..

Clones that are of interest can be stored long term by adding 50 µl of sterile 60% glycerol and freezing at - 80°C.

Note: Once defrosted, clones cannot be refrozen but must be recultured by adding 10µl of vortexed clone culture to 1ml of LB with ampicillin (100µg.ml).

5. Restriction Enzyme Digests

Note: These reagents do not need to be defrosted on ice

10µl PCR product from clone

add 0.5µl restriction enzyme of choice (see table below)

7.5µl sterile water

2µl appropriate buffer (see table below)

Incubate the mix at 37°C for 1 hour and then stop reaction by subsequently incubating at 65°C for 10 minutes preferably in the Gradient PCR machine or in a waterbath.

NOTE: for AluI and RsaI use react buffer I, for HaeIII use react buffer II

Run RFLPs on a 3% agarose gel to visualize with a 25Kb ladder

Restriction Enzyme	Cut site	Reaction conditions
Alu I	3' – TC GA 5 5' – AG CT - 3	37 °C react 1 buffer
Hae III	3' – CC GG 5 5' – GG CC 3	37 °C react 2 buffer
Rsa I	3' – CA TG 5 5' – GT AC -3	37 °C react 1 buffer

Clean-up and send for sequencing

Grow up fresh overnight cultures of the clones to be sequenced and clean up using the Invitrogen SNAP miniprep kit (www.invitrogen.com) and send off for sequencing with the M13F and M13R primers.

6. RNA Work

All equipment (mortars, pestles and spatulas etc) MUST be soaked in 0.01% DEPC (1ml in 1l water) water overnight before use.

DEPC is highly toxic but degrades on autoclaving, waster DEPC water must be autoclaved before disposal down sink.

All solutions must be treated with DEPC if not Rnase/Dnase free. Some solutions are not compatible with DEPC and these must be made up using DEPC treated water

DEPC keeps for 1 month in the fridge

All pipettes must be cleaned with ethanol

Bench must be thoroughly cleaned with ethanol

Use sterile technique at all times and change gloves frequently

DO NOT attempt more than 2 (to 4) samples at a time, speed is critical

β-mercaptoethanol must be added to RLT or RLC buffer before use. 10µl per 1ml buffer.

This is stable for 1 month

RPE buffer must be diluted with 4 volumes of ethanol (96-100%) before use

Columns for RNA cleanup must be kept in fridge

RNA Extraction

Using QIAGEN Rneasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi (www1.qiagen.com) according to manufacturers protocol.

For wood samples: Use <100mg of sample ground to very fine powder using liquid nitrogen

For bacterial cultures: pellet and wash thoroughly in sterile water (if in media).

Resuspend in 450µl buffer (Step 3 of protocol)

Flash freeze in liquid N and incubate for 1-3 mins at 56°C

RNA Cleanup

Using QIAGEN Rneasy Mini Protocol for RNA Cleanup (www1.qiagen.com) according to manufacturers protocol.

DNA Digestion

To make sure all DNA contaminants are removed from the samples. Using QIAGEN Rnase-Free Dnase kit (www1.qiagen.com)

Note: be sure to pipette mix all reagents as they are added and give a quick centrifuge to final mix to ensure.

Set up 2 reactions for each sample (one for subsequent RTPCR and 1 for PCR) and add the following to each:

1µl RNA sample
1µl RDD buffer
2.5µl Dnase I
5.5V sterile water

incubate at 25°C for 15 mins in Mastercycler
add 1µl of 25mM EDTA

incubate at 65°C for 10 mins in Mastercycler

Note: Can stop at this point and store products at – 80°C but preferable to proceed with RTPCR

RTPCR (Reverse Transcription PCR)

Note: RTPCR REAGENTS MUST BE KEPT AT APPROPRIATE TEMPERATURES THROUGHOUT THE PROTOCOL, DEFROSTED AND KEPT ON ICE WHILST DISPENSING. ALSO SPEED IS CRITICAL

A normal PCR is run concurrently with the RTPCR to check for the presence of any DNA not removed during the DNA digest step

For PCR of each RNA sample dispense the following:

5µl PCR buffer
3µl MgCl₂
1µl DNTPs
1µl forward primer EUB933F
1µl reverse primer EUB1387R
0.25µl Taq
27.75µl sterile water
Total 39µl

add 11µl of digested RNA sample

Perform PCR

For RTPCR of each RNA sample, dispense the following:

10µl 5XRTPCR buffer
10µl Q solution
2µl DNTPs
2µl enzyme mix
3µl forward primer EUB933F
3µl reverse primer EUB1387R
9µl Rnase free water
Total 39 µl

Add 11µl digested RNA sample

Perform RTPCR

Visualisation of PCR/RTPCR products

Run 15-20µl of product on 15% agarose gel at 100V and visualise under UV with an Ethidium Bromide stain.

Run the PCR and RTPCR products for each sample alternatively on gel for ease in identifying possible traces of undigested DNA

Recovery of cDNA for cloning

Re-run 2 lanes of each clean cDNA sample on 15% agarose gel

Extract CDNA using QIAEX II Gel Extraction Kit from QIAGEN (www1.qiagen.com), following the manufacturers protocol

Note: always use fresh buffers in the tanks and gels for best recovery of cDNA fragments

Cloning

Using the Invitrogen Kit: TOPO TA Cloning (www.invitrogen.com)

Ligation & Transformation

Use one vial of One Shot cells for every three transformations.

The ligation reaction mixture:

1.00µl Sterile water

0.33µl Salt Solution

0.33µl TOPO Vector

Make up mastermix and aliquot 1.66µl into each reaction tube

Add 0.33 µl of fresh RTPCR product

Note: can use 1.33 µl of product rather than adding water if DNA concentration is known to be low

Gently mix by tapping the tube (*do not* use a pipettor!) and incubate at room temperature for 5 minutes.

Add 16 µl of the One Shot Chemically Competent *E. coli* cells to each cloning reaction.

Incubate on ice for 5 to 30 minutes.

Heat-shock the cells for 30 seconds at 42°C without shaking.

Immediately transfer the tubes to ice.

Add 72 µl of the room-temperature SOC medium.

Incubate tubes horizontally for 1 hour in the 37° shaking incubator at 200 rpm.

Note: do not incubate for longer than 1 hour as this allows for about 3 divisions

Using sterile technique, spread 35 µl (do two plates) from each transformation onto a prewarmed LB plate. Let stand right-side up for about 5 minutes to allow the cells to adhere to the agar, then invert and incubate overnight at 37°C.

NOTE: do not incubate for longer as get an over abundance of false positives as the ampicillin is used up.

Clone Selection & Checking

Pick off 20 white colonies and cÿlture in 1 ml of LB + ampicillin for minimum of 5 hours

200rpm @ 37°C

Take 3 µl of cÿlture in 21.45 µl sterile water and boil for 10 mins

Overnight or Day 2. PCR

3µl Clone Culture

3µl PCR buffer

0.9µl MgCl₂

0.5µl dNTPs

0.5µl Primer M13F

0.5µl Primer M13R

0.15µl Taq

21.45µl Sterile water

TOTAL 30 µl

PCR profile as recommended for DNA cloning (section 5 above)

visualise using agarose gel electrophoresis (8 or 10 µl on 1.5% gel, 1kb marker, 1-1.5 hours @ 80V). Successful clones should be approx. 400-600bp in size whereas false positives will yield different sized products.

Restriction Digest and Subsequent Clean up and Sequencing

As with DNA see section 5 above.

7. Phylogenetics

Sequences from isolates, environmental samples, DGGE bands and clone libraries were aligned with those representing known bacterial taxa deposited in databases. Alignments were constructed using CLUSTAL X and manually adjusted in text editors (Se-Al and PAUP). Data matrices representing the major bacterial groups were prepared using 50 to 500 taxa for 1000 nucleotides. These matrices were used to perform phylogenetic analyses under parsimony and distance criteria implemented in PAUP. Analyses were performed on unrooted data and when *Aquiflex pylophilus* was used as the outgroup. Under general parsimony criterion, heuristic searches were made with random sequence addition for 10 replicates and trees constructed using the TSB algorithm. Genetic distances were calculated using the Kimura-2, log-det, GTR and maximum likelihood evolutionary models and trees constructed via the nearest neighbor method.

8. FISH

NOTE: *in situ* hybridisation is a very sensitive technique, it is important that all reagents used must be free from all microbes. All reagents must be filtered through a 0.2 µm pore size filter into a clean bottle prior to autoclaving (for those reagents that can be autoclaved). As an insurance step, filter all reagents again immediately prior to use.

Fixation of material

NOTE: Gram-negative bacteria are fixed in paraformaldehyde ("PFA ") and gram-positive bacteria in ethanol but both types can be fixed simultaneously in PFA if a lysozyme treatment is used. The structure of cells fixed with just ethanol is not maintained very well. The fixation protocol used will need to be tested for each sample or each individual gram-positive strain.

Reagents and solutions

1x PBS

130 mM NaCl (3.8 g per 500ml)

10 mM Na₂HPO₄ ·H₂O (1.79 g per 500ml)

3 mM NaH₂PO₄ ·H₂O (0.23 g per 500ml)

pH should be 7.2, adjust with NaOH or HCl as necessary

Filter through 0.22 µm filter into a sterile container, autoclave and store at 4°C.

3 x PBS

390 mM NaCl (11.4 g per 500ml)

30 mM Na₂HPO₄ ·H₂O (5.37 g per 500ml)

9 mM NaH₂PO₄ ·H₂O (0.69 g per 500ml)

pH should be 7.2, adjust with NaOH or HCl as necessary

Filter through 0.22 µm filter into a sterile container, autoclave and store at 4°C.

4% paraformaldehyde ("PFA") in PBS

Heat (to ~ 60°C) 33 ml of high purity water (i.e. MilliQ water) that has been filtered.

Add 2 g of paraformaldehyde (work in fume cupboard!) and one drop of 2 M NaOH, and stir for about 2 min when the PFA should go into solution.

Add 16.5 ml of 3 x PBS that has been filtered and cool the resulting solution on ice (if you are in a hurry).

Adjust the pH to 7.2 with NaOH or HCl as needed.

Store at 4°C and use within 24 hours or aliquot into small volumes (750 µl is a very convenient volume) and store at -20°C (keeps indefinitely). NOTE: the final solution should not be filtered or autoclaved.

Oxalic acid
make a 500 mM (31.52 g per 500ml MilliQ water) solution and filter (0.22 µm).

Blocking Reagent

Roche Diagnostics cat. No. 1096176 (50g)

Procedure

Take one ml of sample (sample size can be adjusted to suit needs, aim for 10^6 - 10^9 cells/ml) and centrifuge at 13000 rpm for 5 min.

Resuspend cells in 250 µl of ice-cold PBS.

Mix this suspension with 750 µl PFA fixative and incubate at 4°C for 1-3 h.

If fixing a cell suspension that contains significant amounts of iron (Fe^{2+} or Fe^{3+}), a precipitate of iron phosphate will form. To remove this, add the oxalic acid solution in small volumes until the solution clears and proceed. Otherwise go step 5.

Pellet cells, thoroughly remove supernatant and wash the cells (use pipette to resuspend) in 1 ml PBS. Pellet cells again.

Resuspend cells in PBS to a concentration of 10^8 - 10^9 cells/ml and add one volume of ice cold ethanol and mix (most likely just resuspend back to ½ of the original volume with PBS and add the same amount of ethanol).

Fixed cells can be stored at -20°C for several months (most likely years). Cells are ready to be spotted onto slides for FISH.

Preparation of microscopic slides

Reagents and solutions

Ethanolic KOH

95 ml ethanol

5 ml deionized (MilliQ) water that has been filtered (0.2 µm)

10 g KOH (this may not dissolve completely but not to worry it is only a cleaning solution).

Gelatine solution

0.15 g gelatine

0.02 g chromium potassium sulfate 12 hydrate [$CrK(SO_4)_2 \cdot 12H_2O$]

200 ml deionized (MilliQ) water that has been filtered (0.2 µm) and heated to 70°C prior to use.

Ethanol series

3 ethanol solutions of 50% (v/v), 80% (v/v) and 95% (v/v) made with deionized (MilliQ) water that has been filtered (0.2 µm).

Lysozyme Solution

25 µg lysozyme/ml filtered deionized water (our lysozyme has an activity of 41,100 Units/mg solid, so we are using 1027.5 U/ml)

Procedure

NOTE: Do as many as possible as they can be stored in the fridge (4°C) for up to 6 months and warm slides to room temperature before starting

Clean slides by soaking in ethanolic KOH for 1 hour.

Rinse slides well in deionized (MilliQ) water that has been filtered (0.2 µm) and let dry.

Place slides in gelatine solution at 70°C and hold horizontally for about 10 seconds to evenly coat the slides. Let the excess solution drain back into container. Allow the slides to dry and either use immediately or store in a sealed slide box at 4° C until needed.

Spread 5 to 10 µl of fixed sample (diluted with filtered deionized water if necessary) on to a gelatine coated slide (can fit 2 samples per slide if you are careful not to allow cross-contamination) and allow the smears to air dry.

Lysozyme step

Place slide at 4C for 10 min to equilibrate at this temperature.

Add a 10 µl of lysozyme solution to each smear and incubate at 4C for 10 min.

Rinse well by immersing the slide into filtered deionized water two times.

Dehydrate the sample smears by immersing slide into a series of solutions of 50%, then 80% and then 95% ethanol, for 3 min in each.

Let slide completely dry.

The slide is ready for immediate hybridisation or can be stored in the dark at room temperature indefinitely.

Fluorescent *In Situ* Hybridisation (FISH):

NOTE: during the hybridisation process, avoid exposing the labelled probes to excess light by performing this entire hybridisation procedure in a darkened room (not completely without light) and storing the hybridised slides in the dark.

Reagents and solutions

NOTE: Autoclave all stock solutions and use to prepare hybridisation and wash buffers. Use deionized water such as MilliQ water (unless otherwise specified) and filter these solutions with a 0.2 µm pore size filter immediately before addition of formamide and use.

1 M Tris/HCl pH 7.4 (60.505g Tris Base, 440ml H₂O, 60ml HCl)

5 M NaCl (146.1g NaCl in 500ml H₂O)

10% SDS (40g SDS in 360ml H₂O adding HCl dropwise to make 400ml)

Deionized formamide (kept at -20 °C in small aliquots (1ml). **CARE: handle with gloves**

5 mM EDTA

add 36.53 g EDTA (= Na₂ ethylenediaminetetra-acetic acid) to 200 ml dH₂O

adjusting the pH to 8.0 with 10 M NaOH, the solution will clear when you approach pH 8 when the EDTA is dissolved make up to 250 ml with deionized H₂O and autoclave before use

DAPI solution

1 mg 4',6-diamidino-2-phenylindole (DAPI)/ml deionized water that has been filtered and autoclaved. Store in a microfuge tube at -20°C.

Mounting medium

Dissolve 0.233 g DABCO in 2.5 ml water, add 500 µl of 1 M Tris (pH 9.5) and mix well, and finally add 7 ml (8.75 g) glycerol and mix thoroughly. Store this in small aliquots at -20°C.

If you needed, the glycerol concentration can be altered to match the refractive index of the immersion oil.

NOTE: The amount of glycerol can affect the refractive index of the immersion oil so move up or down percents if this is a problem

Procedure

Prepare 2 ml of hybridisation buffer according to required stringency (see Table 1 below) by adding the appropriate amount of deionized formamide.

Place a tissue, soaked with about 1.8 ml of the hybridisation buffer, in a 50 ml conical test tube. Allow the tube to equilibrate in the hybridisation oven set at 46°C for about 30 min.

NOTE: Probes and material containing probes must always be in the dark

Add 10 µl of hybridisation buffer containing 25 ng of each labelled probe used to each sample smear and cover with a coverslip.

Transfer slide, smear side up, to the equilibrated tube in the hybridisation oven and incubate at 46°C for 2 h.

Gently removed the cover slip and immerse slide for 15 min in pre-warmed (48°C) wash buffer of appropriate stringency (see Table 2) at 48°C.

Gently rinse the slide with filtered deionized water from a squirt bottle and allow the slide to dry.

Add 1 µl of stock DAPI solution to 1ml mounting medium and smear 10µl on slide. Add a coverslip and incubate in the dark for 10 min. For long term storage, you can seal the coverslip with clear nail polish (that does not fluoresce) and keep the slide in the dark.

Preparation of hybridisation buffer

To prepare 2 ml of the hybridisation buffer mix the following and filter (0.2 µm) immediately prior to addition of formamide (or use filtered ingredients)

40 µl 1 M Tris-HCl pH 7.4

2 µl 10% SDS

360 µl 5 M NaCl

X µl formamide (see below)

Y µl deionized (MilliQ) water (see below)

Table 1. Preparation of hybridisation buffer to the appropriate stringency, for hybridisation at 47°C in the presence of 0.9 M cations (i.e. Na⁺), by addition of formamide. The addition of formamide to change stringency allows hybridisation reactions to be carried out at a constant temperature when using different oligonucleotide probes. See section below for required amount of formamide

% Formamide	X µl formamide	Y µl deionized water
0	0	1598
5	100	1498
10	200	1398
15	300	1298
20	400	1198
25	500	1098
30	600	998
35	700	898
40	800	798
45	900	698
50	1000	598
55	1100	498
60	1200	398

Preparation of washing buffer

To prepare 50 ml washing buffer mix the following and filter (0.2 µm) immediately prior to use.

39.5 ml deionized water

50 µl 10% SDS

1 ml 1 M Tris-HCl, pH 7.4

0.5 ml EDTA

X µl 5 M NaCl (see below)

Y µl deionized water (see below)

Table 2. Preparation of washing buffer to appropriate stringency by altering the sodium chloride concentration to match that achieved in the hybridisation buffer by formamide (see Table 3 for required amount of formamide needed for probes). That is, for each 5% formamide used in the hybridisation buffer, you must decrease the NaCl in the wash buffer by 29.3%.

% formamide used in hybridisation buffer	NaCl (M) needed in wash buffer	X µl of 5 M NaCl to add	Y µl H ₂ O to add
0	0.9	9000	0
5	0.636	6360	2640
10	0.450	4500	4500
15	0.318	3180	5820
20	0.225	2250	6850
25	0.159	1590	7410
30	0.112	1120	7880
35	0.080	800	8200
40	0.056	560	8440
45	0.040	400	8600
50	0.028	280	8720
55	0.020	200	8800
60	0.014	140	8860

Epifluorescence Microscopy

Samples were viewed under a Nikon Eclipse E800 epi-microscope using the appropriate filter sets:-

For DAPI

UV1A Excitation 365/10 i.e. range of 360-370nm

Dichroic Mirror (DM) long pass barrier at 400nm

Barrier emission filter (BA) at 400nm

For Fluorescein

UV1A Excitation 460-500nm

DM barrier at 505nm

BA barrier at 510nm

For CY3

UV1A Excitation 510-560nm

DM is a barrier at 565nm

BA 570-610nm filter

9. PRIMER Statistical Analysis

The text below is a personal interpretation of information provided by the developers of the PRIMER software package. All information is taken from the following 2 sources:

Clarke, K.R., Gorley, R.N. PRIMER v5: User Manual/Tutorial. PRIMER-E: Plymouth. (2001).

Clarke, K.R., Warwick, R.M. Change in marine communities: an approach to statistical analysis and interpretation. Plymouth: Plymouth Marine Laboratory, 144pp. (1994).

PRIMER is a multivariate statistical package specifically developed for the analysis of complex ecological data. The underlying strategy is to highlight any patterns of similarity or dissimilarity across samples of data on the basis of their biotic composition. The results can then be compared with known or hypothesised interrelations between groups of samples, in the case of the BACPOLES research the predetermined groups are samples taken from different wood types and different regions.

PRIMER operates by comparing the extent to which two or more samples share particular taxa at similar levels of abundance. It does this by calculating a similarity coefficient (S) between every pair of samples. Similarity coefficients define a range of 0 to 100% (or 0 to 1) where S=0 when two samples have no species in common and S=100% (or 1) when two samples are identical. The most commonly used algorithm for calculating similarity coefficients is the Bray-Curtis coefficient and that has been used here. The qualities of this coefficient that make it useful for working with ecological data are that it is independent of both scale changes (*i.e.* whether data is collected per m² or per cm²) and joint absences of species from the data (*i.e.* if S is dependent on the joint absence of a species then one could say that alpine and desert communities are similar because blue whales are found in neither of them!). A similarity matrix is constructed from the comparison of every pair of samples and from this a number of graphical representations of the data can be made through hierarchical clustering and ordination techniques.

Once patterns of similarity or dissimilarity between groups of samples have been identified, it is desirable to be able to test for these differences. PRIMER offers a routine, ANOSIM (analysis of similarities), which is analogous to the ANOVA (analysis of variance), a tool used for the analysis of univariate data. ANOSIM generates a test statistic (R value), which falls between 1 and 0. If R=1, then all replicates within a group of samples are more similar to each other than any replicates from different groups of samples. If R=0 then the similarity within and between groups of samples are on average the same. An important note to stress is that ANOSIM can only be applied to groups of samples that have been specified apriorly, *i.e.* before any data has been analysed.

PRIMER can also be used to identify taxa that are influential in characterising the biotic composition of groups of samples. The SIMPER (similarity percentages) routine is used to calculate the percentage contribution that each species makes to the similarity within groups of samples, based on similarity coefficients. Typical taxa that contribute highly to the similarity within groups of samples do so consistently across samples and generally in high abundance. SIMPER can also calculate the overall percentage contribution that each species makes to the average dissimilarity between groups of samples and in much the same way as outlined above, can highlight species that may be considered good discriminators between groups of samples.

SIMPER can be used to rapidly identify differences in patterns of species distribution and abundance between samples, hence its usefulness to the BACPOLES research.