**IDENTIFICATION FOR NORTH ATLANTIC *PORPHYRA SENSU LATO* AT THE GENUS AND SPECIES LEVELS USING RESTRICTION ENZYMES.**

## *Background:*

*Porphyra* are an ancient group of red algae which are part of red algal order Bangiales. Recently the genus *Porphyra* has undergone a major taxonomic revision breaking it into eight leafy genera and seven filamentous genera [1]. *Porphyra sensu lato*, means everything that used to be called *Porphyra*. The combination of DNA sequences of the nuclear small subunit (SSU) of the ribosome and plastid ribulose bisphosphate carboxylase oxygenase large subunit gene (rbcL) were instrumental in distinguishing species and identifying major taxonomic groups.

The haploid leafy thalli are found in the rocky intertidal all over the globe. *Pyropia* was recently separated out as its own genera from *Porphyra. Pyropia* species in this genera are cultivated in Asia as a economically important food crop; much of the harvest is used to make the familiar crisp and salty nori wrappers of sushi. Various *Porphyra* and *Pyropia* species are foraged by many cultures around the world including: Asian communities, Western Europeans, and Native Americans; these wild-collected algal foods have many common names such as: laver, slake, black weed, gim, or zicari. More than 150 different species in seven genera have described; these are differentiated based on where the algae are been found, their seasonality, and the morphology of reproductive tissue [2]. However identifying species has always been difficult: the algal morphology is "plastic" responding to variable ocean and habitat conditions and many species have overlapping diagnostic features (Figure 1). Sexual tissue producing male or female spores assists with identification, but reproductive tissue is not always present.

Figure 1. A formerly cryptic winter ephemeral "*Porphyra*" from Dover Pt. NH described by West *et al*. Nova Hedwigia 80: 1-24, 2006; this cryptic species is now identified as *Pyropia yezoensis*

The leafy tissue ("blade") is either one or two cell layers thick, greenish brown to red in color, and ranges from being long and narrow to round and even ruffled. Reproductive cells, when present, are found along the margins of the blade. Male and female reproductive tissue can be intermixed or segregated into patches or even opposite sides of the blade. The leafy red algae represent the haploid phase of the algal life cycle. These algae also have a separate diploid phase, a microscopic filamentous form that bores into calcareous substrates such as oysters or barnacles (Figure 2).

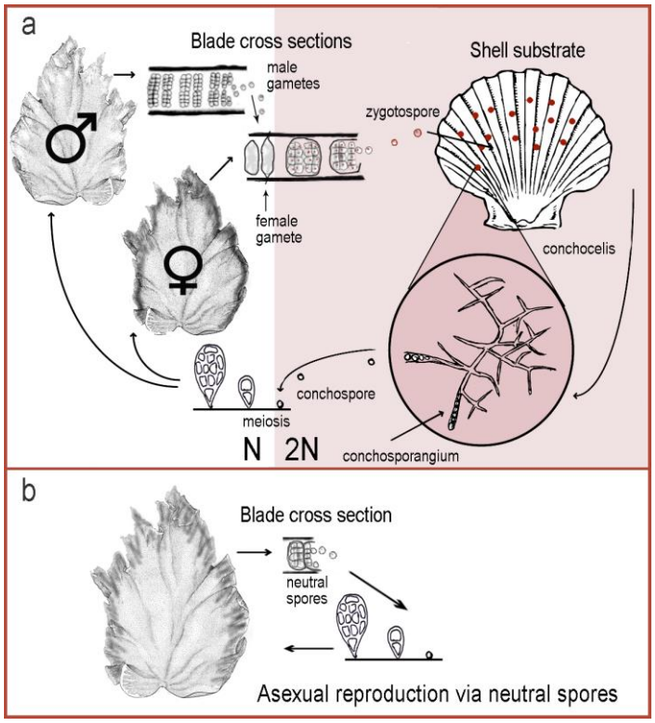


Figure 2 Heteromorphic, sexual life history of *Porphyra* spp.; both phases of the life history produce spores that regenerate each phase directly (A) and reproduction of the blade by an asexual pathway involving neutral spores (B, major emphasis of the *Porphyra umbilicalis* genome project). [3]

A restriction fragment length polymorphism (RFLP) assay, based on inter-specific sequence variation was developed to provide a simple tool for screening and sorting large collections of *Porphyra sensu lato* from the Northwest Atlantic (10-12 species in several different genera). The assay begins with DNA amplification of the *ribulose bisphosphate carboxylase oxygenase large subunit* (*rbc*L) gene and the *rbc*L-*rbc*S intergenic spacer. The DNA is cut with restriction enzymes: these are endonucleases; that is the enzyme cuts within a DNA molecule at a specific sequence (http://www.dnalc.org/resources/animations/restriction.html). When *rbc*L is digested with a single restriction enzyme (*Hae* III), the resulting DNA fragments are separated by size http://www.dnalc.org/resources/animations/gelelectrophoresis.html. The sizes of the fragments produced are specific for each multiple Bangiales species in the Northwest Atlantic including several cryptic taxa. An additional enzyme (*Hind* III) was necessary to distinguish between the sister species *Pyropia leucosticta* and *Pyropia yezoensis*.

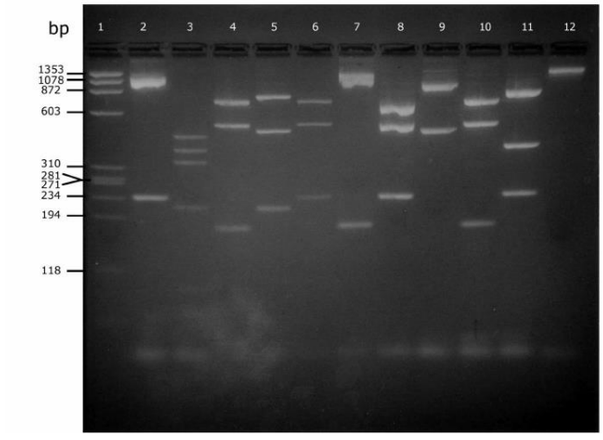


Figure 3 RFLP patters of the *rbc*L + *rbc*L-*rbc*S spacer using the *Hae*III restriction enzyme. Lane 1ϕX174 ladder (bp sizes in margin), Lane 2= *Wildemania amplissima*, Lane3= *Porphyra diocia*, Lane 4=*Pyropia leucosticta*, Lane 5= *Porphyra linearis*, Lane 6= *Wildemania miniata*, Lane 7 *Porphyra purpurea*, Lane 8= *Pyropia suborbiculata*, Lane 9= *Porphyra umbilicalis*, Lane 10 *Pyropia yezoensis*, Lane 11=*Boreophyllum birdiae.* The diffuse band at the bottom of each lane is excess primer. Modified from[5]

## Lab experiments*:*

GENERAL PRECAUTIONS

- Wear gloves; change gloves if they get dirty.
- Keep tubes on ice. Keep caps to tubes closed as much as possible.

*Many of the tools and reagents are common to high school and undergraduate biology teaching labs.*

REGENTS AND EQUIPMENT REQUIRED:

1. Freshly collected *Porphyra* (if you live near the ocean) or order from Maine Sea Coast Vegetables (MCSV; Laver "Wild Atlantic Nori" https://www.seaveg.com)
2. Qiagen DNasy Plant Mini-Kit (http://www.qiagen.com/products/genomicdnastabilizationpurification/dneasyplantsystem/dneasyplantminikit.aspx? ).
3. Agarose Gel electrophoresis supplies
    a. Several vendors (i.e. Invitrogen.com) sell precast gels
    b. BioRad sells a STEM electrophoresis classroom kit.
    c. If you school has electrophoresis equipment, you will need to buy agarose, Tris Borate Electrophoresis Buffer and DNA ladders (www. Promega.com: 1kb ladder G5711, 100 bp DNA Ladder G2101)
4. High Speed microcentrifuge
5. Pipetmen® http://www.pipetman.com/1ml, 200 µl, 20 µl (mechanical micro-pipettors)
6. DNA thermo cycler
7. Mortar and Pestles
8. Oligonucleotide primers for polymerase chain amplification of *rbc*L-*rbc*S for *Porphyra sensu lato* may be ordered from www. Invitrogen.com
    a. Forward Primer F67 (5'-TACGCTAAAATGGGTTACTG) was developed from overlapping sequence of an earlier universal *rbc*L primer F57 outlined by [4]..
    b. Reverse Primer rbc-spc (5'-CACTATTCTATGCTCCTTATTKTTAT) was designed to selectively amplify *Porphyra* species [5].
    c. The primers are inexpensive (20-30 $), and if stored frozen, are good for several years and hundreds of reactions.
9. GoTaq green master mix for Polymerase Chain Reaction (PCR) . (www.Promega.com; M7911)
10. Other disposables:
    a. DNeasy Plant Mini Kit (www.qiagen com, catalog 69104)
    b. Microcentrifuge tubes( http://www.usascientific.com/Seal-Rite-2.0-ml-tube.aspx; 1620-2700)
    c. PCR strip tubes (http://www.usascientific.com/0.2ml-pcr-8-tube-strip-flat-8-cap-strips.aspx; 1402-2500)
    d. Aerosol Resistant pipette tips (http://www.usascientific.com/TipOne-filter-tips.aspx)
    e. 6X Gel loading dye (www. Promega.com, G1881)
    f. Restriction enzymes:
        i. *Hae* III (www. Promega.com; R6175)
        ii. *Hind* III (www. Promega.com; R6041)

## *Lab 1: DNA extraction from seaweed*

DNA is extracted either from freshly collected or dried algae (e.g. Maine Sea Coast Vegetables' laver). The Qiagen DNeasy Plant Mini-Kit is used to extract DNA. One of the benefits of the proprietary kit technology is that the protein/lipid/polysaccharide precipitation step uses non-hazardous reagents.
Follow the directions in the Qiagen DNeasy kit to extract DNA from all samples.
Store DNAs at 4 degrees Centigrade for short intervals (less than one week) or frozen at -20 degrees Centigrade

### GEL ELECTROPHORESIS TO ESTIMATE DNA YIELD (OPTIONAL)

Use gel electrophoresis to estimate the amount of DNA you recovered. Load 5 µl of DNA extract onto a 0.8 % agarose gel, using supplies and equipment available from Carolina Biological Supplies (DNA Gel Electrophoresis' http://www.carolina.com/biotechnology-teaching-resources/dna-gel-electrophoresis/10123.ct) or Load 0.5 µg of standard for comparison(1 kb DNA ladder) with loading buffer. Run electrophoresis in TBE buffer at 5 volts per cm gel. Use gel green and a black light to visualize the DNA on the gel and estimate the amount extracted by comparison to known amount of the molecular ladder standard.

### PREPARE DILUTIONS OF DNA EXTRACTS

1. Fill an ice bucket with ice.
2. Thaw your extracts (Lab #1) with gloved hands/ice/…
3. Vortex your extract gently or flick the tubes with your finger to re-suspend the DNA.
4. Microcentrifuge your DNA extracts briefly (balance the tubes) to bring all solution back to the bottom of the microcentrifuge tube. Put tubes on ice.
5. Prepare a 1:10 and/or a 1:100 and/or a 1:300 dilution of each tissue extract (label tubes as you go). For example, for the 1:100 dilution, take 4 µL of the extract (using the appropriate pipetman and small tips) and add it to a tube that contains 396 µL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). It is best to make this volume of the dilution (Why?)
6. Repeat steps 3 and 4 with each sample that needs to be prepared as a dilution. Keep tubes on ice when not working with them.

### AMPLIFYING THE *rbc*L GENE + SPACER FROM YOUR DNA EXTRACT USING PCR

http://www.dnalc.org/resources/animations/pcr.html

#### *Prepare the PCR cocktail*

The Promega GoTaq Master Mix already includes most of the components of the PCR reaction (see handout). However, you'll have to add primers and your DNA.

**PCR Recipe:**

| | |
|---|---|
| GoTaq Green Master Mix[1] (2x) | 25 µl |
| Forward Primer (FP 10 µM) | 5 µl |
| Reverse Primer (RP 10 µM) | 5 µl |
| Nuclease-free $H_2O$ | 10 µl |
| DNA template (your sample) | 5 µl |
| TOTAL Volume of reaction | **50 µl** |

1. Each cocktail (CKT) final volume (GoTaq Green Master Mix + Forward Primer + Reverse Primer) will be 45 µL (and then you'll add an appropriate DNA template to each cocktail for a reaction volume of 50 µl.
2. Mix enough Master Mix, FP, and RP to have 4 tubes worth for each primer set. Keep your mixtures on ice. Do this by using one microcentrifuge and making four times more cocktail than what is needed for each single reaction.

[1] http://www.promega.com/resources/protocols/product-information-sheets/g/gotaq-green-master-mix-m712-protocol/

3. Pipet (use 100 µL pipetman) CKT into each tube in a PCR strip (there will be 8 tubes/PCR strip). Keep strip on ice.
4. Add 5 µL of your DNA extract to each PCR tube after 45 µL of CKT are in the tube. Cap the strip. Label the strip top (initials, tube numbers).
5. Gently vortex capped strip and spin down before putting into the thermal cycler.

Put the tubes into the thermal cycler ("PCR machine").

*Amplification Program*: The amplification profile began with an initial denaturation step of 93 °C for 3 min and was followed by 29 cycles of 30 sec at 93 °C, 1 min at 45 °C, and 1.5 min at 72 °C. (2-3 hours)

USE GEL ELECTROPHORESIS TO CONFIRM THE PCR WORKED:

Separate fragments on a 2% agarose gel in either Tris Borate EDTA (TBE) buffer, or Tris Acetate EDA (TAE) buffer at ~5 volts/cm of gel. Use PCR ladder standards, and estimate yield. For the next step, you need at least 0.1 µg of DNA per µl of PCR product for restriction fragment analysis.

## *Lab 2: Restriction digest and sizing DNA fragments*

RESTRICTION DIGEST ANALYSIS.

1. Pipet 25 µl of each PCR product into a fresh, labeled microfuge tube[2] and place on ice.
2. Add 0.5 µl *Hae* III enzyme or *Hind*III to the tube; use a fresh pipet tip for each digest.
3. Cap and gently mix the restriction reaction; incubate at 37°C for 10-15 minutes.
4. Add 5 µl loading dye to each sample.
5. Load each sample on a 2% agarose gel, with 100 bp DNA standard ladder in one lane.
6. Run gel with either TAE or TBE buffer at ~5 mV/cm of gel. Run electrophoresis till the bromophenol blue band reaches just reaches to the bottom.
7. To estimate fragment sizes, generate a standard curve using semilog graph paper. Plot the distance from the well against the log molecular weight for each band in the standard[3]. From this graph you can calculate an equation log molecular weight (y)=- mx + b, where –m is the slope of the linear part of the curve, and x is the distance the fragment has moved from the loading well.

   From the calculated size of your *Hae*III restriction sites, identify which genus/species your sample represented:

| **Taxon** | **Hae III fragments** | **Hind III fragments** |
|---|---|---|
| *Boreophyllum birdiae* | 237 395 849 | 1481 |
| *Porphyra dioica* | 99 216 337 382 447 | 1481 |
| *Porphyra linearis* | 216 482 783 | 101 1380 |
| *Porphyra purpurea* | 179 1302 | 101 1380 |
| *Porphyra umbilicalis* | 482 999 | 101 1380 |
| *Pyropia katadae*[4] | 1481 | 1481 |
| *Pyropia leucosticta* | 58 179 521 723 | 1481 |
| *Pyropia suborbiculta* | 237 533 711 | 194 1287 |
| *Pyropia yezoensis* | 58 179 521 723 | 538 943 |
| *Wildemania amplissima* | 237 1244 | 1481 |
| *Wildemania cuneiformis* | 237 521 723 | 626 855 |

[2] You can incubate 0.2 ml PCR tubes in the thermocycler or standard 1.5 ml tubes in a water bath at 37 °C.
[3] See http://www.phschool.com/science/biology_place/labbench/lab6; this LabBench activity demonstrates each step in the DNA restriction fragment analysis.
[4] Predicted based on GenBank Accession HQ728199.1

*Microscopic analysis.*
If the blades your samples came were reproductive, it may be possible to find spermatangia (male gametes) or zygotosporangia (fertilized eggs = zygotes) or an asexual spore (e.g., neutral spores in *P. umbilicalis*). After you've made a tentative ID based on RE analysis, go back to your saved sample from Lab 1 and examine the tissue. If the edge looks red or pale yellow, use water to soften a small piece of the MCSV blade you extracted DNA from, and then cut cross sections and surface sections to look for reproductive cells. Examine your mounted slide at the compound microscope.

## *References:*


1. Sutherland JE, Lindstrom SC, Nelson WA, Brodie Jet al. 2011. A New Look at an Ancient Order: Generic Revision of the Bangiales (Rhodophyta). J Phycol 47:1131-1151.

2. Guiry MD, Guiry GM. AlgaeBase. National University of Ireland, Galway; 2011.

3. Blouin NA, Brodie JA, Grossman AC, Xu Pet al. 2011. *Porphyra*: a marine crop shaped by stress. Trends in Plant Science 16:29-37.

4. Hommersand MH, Fredericq S, Freshwater DW. 1994. Phylogenetic systematics and biogeography of the Gigartinaceae (Gigartinales, Rhodophyta) based on sequence-analysis of rbcL. Bot Mar 37:193-203.

5. Teasdale B, West A, Taylor H, Klein A. 2002. A simple restriction fragment length polymorphism (RFLP) assay to discriminate common *Porphyra* (Bangiophyceae, Rhodophyta) taxa from the Northwest Atlantic. J Appl Phycol 14:293-298.

6. Promega Manufacturer's Instructions for GoTaq Master Mix