

Lab Activity: Identification of North Atlantic *Porphyra sensu lato* at the genus and species levels using restriction enzymes

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Learning Objectives

By the end of this activity, students should be able to distinguish between morphological taxonomy and molecular -assisted taxonomy

By the end of this activity, students should be able to use restriction enzymes to probe for DNA sequence variation.

By the end of this activity, students should be able to design an assay to distinguish cryptic species using DNA variation in moderately conserved genes.

Assessment Method

A student will show they have mastered the learning objective when they can successfully describe the differences between morphological taxonomy and molecular-assisted taxonomy. (Bloom scale: Interpreting)

A student will show they have mastered the learning objective when they can successfully predict the size and number of restriction fragments produced from *rbcL* amplicons for Northwest Pacific *Porphyra sensu lato* by a common restriction enzyme (Bloom scale: Analyzing) *Pyropia abbottiae*, *Wildemania schizophylla*, *Pyropia gardneri*, *Pyropia pulchra*¹(Lindstrom, S.C. J Appl Phycol (2008) 20: 951. doi:10.1007/s10811-008-9313-9)

A student will show they have mastered the learning objective when they identify a moderately conserved gene for which DNA sequences are available in GenBank, to distinguish cryptic species (i.e. *Ulva*, *Enteromorpha*²) in the same genus or family, using PCR amplification and analysis either DNA sequencing or RFLP analysis (Bloom scale – creating, evaluating) [*Ulva lactuca*, *U. rigida*, *U. compressa*, and *U. pertusa*]

Instructor Notes

Materials or supplies 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(<https://www.qiagen.com/us/shop/sample-technologies/dna/genomic-dna/dneasy-plant-mini-kit/#orderinginformation>).
3. Mortar and Pestle 4”, chilled; small stainless-steel spatula (i.e. Fisherbrand™ Spoonula™ Lab spoon
4. Ice and ice buckets

¹Taxa names updated according to Sutherland et al. 2011

² *Enteromorpha* has been synonymized with *Ulva*: Hayden, H.S., Blomster, J., Maggs, C.A., Silva, P.C., Stanhope, M.J. & Waaland, J.R. (2003). Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology* 38(3): 277-294.

5. Oligonucleotide primers for polymerase chain amplification of rbcL-rbcS for *Porphyra sensu lato* may be ordered from [www. Invitrogen.com](http://www.invitrogen.com).

a. Forward Primer F67 (5'-TACGCTAAAATGGGTTACTG) was developed from overlapping sequence of an earlier universal rbcL primer F57 outlined by Teasdale et al. 2002].

b. Reverse Primer rbc-spc (5'-CACTATTCTATGCTCCTTATTKTAT) was designed to selectively amplify *Porphyra* species [5].

c. Notes: The primers are inexpensive (\$ 20-30), and if stored frozen (in aliquots for each year), are good for several years and hundreds of reactions.

6. GoTaq green master mix for Polymerase Chain Reaction (PCR) . (www.Promega.com; M7911)

7. Agarose Gel electrophoresis supplies

a. Several vendors (i.e. [Invitrogen.com](http://www.invitrogen.com)) sell precast gels

b. If your school has electrophoresis equipment, you will need to buy:

i. Electrophoresis grade agarose,

ii. Tris Borate Electrophoresis Buffer and

iii. DNA ladders ([www. Promega.com](http://www.Promega.com): 1kb ladder G5711, 100 bp DNA Ladder G2101)

6. Other Disposables

a. . DNeasy Plant Mini Kit (www.qiagen.com, catalog 69104)

i. Buffer AP1 and AW1 concentrate may form precipitates upon storage; if necessary warm to 65°C to redissolve. Cool briefly then add 96-100% ethanol as indicated on the bottle to dilute AWI to working strength. Do not heat AWI after adding ethanol.

ii. Add ethanol to Buffer AW2 concentrate to working strength.

b. Microcentrifuge tubes 1.5 ml and 2.0 ml (<http://www.usascientific.com/Seal-Rite-1.5-ml-tube.aspx> . 1615-5500; [http://www.usascientific.com/Seal-Rite- 2.0-ml-tube.aspx](http://www.usascientific.com/Seal-Rite-2.0-ml-tube.aspx); 1620-2700)

c. PCR strip tubes (http://www.usascientific.com/pcrtubesstrips_1.aspx; 1402-2500)

d. Aerosol Resistant pipette tips (<http://www.usascientific.com/TipOne-filter-tips.aspx>)

e. 6X gel loading dye ([www. Promega.com](http://www.Promega.com), G1881)

f. Vinyl or latex disposable gloves

g. Restriction enzymes:

i.Hae III ([www. Promega.com](http://www.Promega.com); R6171)

ii.Hind III ([www. Promega.com](http://www.Promega.com); R6041)

h. PCR grade water (purified, double distilled, deionized, autoclave W1754 Sigmaaldrich.com)

g. Ethanol 96-100% [not denatured ethanol]

h. Ruler and semilog graph paper

i. razor blades

j. glass microscope slides

k. Black, fine point Sharpie pens to label all tubes.

l. 1 kb DNA ladder Promega.com G6941.

m. GelGreen™ Carolina.com Item 217305

n. Safety glasses (VWR 89187-984) or UV face shield

Equipment required:

1. Laboratory balance accurate to 10 mg.
2. Electrophoresis apparatus with Power Supply
 - a. BioRad sells a STEM electrophoresis classroom kit. STEM Electrophoresis Kit. 1665090EDU “4 reference dyes, dye extraction solution, microcentrifuge tubes), TAE buffer, agarose, electrophoresis cell, electrodes, electrical leads, combs, curriculum, for 8 student workstations; ww.bio-rad.com
 - b. PowerPac™ Basic power supply 1645050 (will run up to four electrophoresis gels at one time) or construct a “tower” of 9 volt batteries(http://www.bio-rad.com/webroot/web/pdf/lse/literature/Bulletin_6288.pdf) for each gel box.

OR

 - c. Carolina Biological Supplies also sells a package containing an electrophoresis power supply and gel electrophoresis chambers that runs 4 gels at a time. www.carolina.com Item #213602
3. High Speed microcentrifuge: [Model 16K Microcentrifuge - 1660602EDU](#)
4. Two water baths or heating blocks with thermometers, prewarm to 65°C and 80°C Biorad.,com Digital Dry Bath, 115V 1660562EDU
5. Vortexer; Biorad.com BR20000, 1660610EDU
6. Pipetmen® www.gilson.com 1ml, 200 µl, 20 µl, 10 µl (or other mechanical micro-pipettors)
7. DNA thermo cycler Bio-rad T100™ Thermal Cycler #1861098EDU qualifies for education discount.
8. 254 nm UV transilluminator or Dark Reader™ or Visi-Blue (Blue LED light box)
9. smart phones are adequate to photograph the gels.

Techniques required (those which are not taught during the activity but students must already have a working knowledge):

Assign online modules to cover (or review) before class

- a. DNA amplification by the Polymerase Chain Reaction: <https://www.dnalc.org/resources/animations/pcr.html>
- b. DNA restriction (sequence specific cleavage): <https://www.dnalc.org/resources/animations/restriction.html>
- c. Gel Electrophoresis: separation of DNA by size: <https://www.dnalc.org/resources/animations/gelectrophoresis.html>

Time required: Three lab periods ~ (2 hours each)

1. DNA isolation: ~45 minutes; gel electrophoresis (0.8% agarose gel) to determine yield ~1 hour;
DNA amplification by PCR: set up 15 minutes; amplification ~2 hours (outside of class) store at 4 °C.
2. Gel electrophoresis (2% agarose) to confirm amplification, restriction digest 60 minutes, ’
3. Gel electrophoresis to measure HindIII fragment sizes ~1 hour and analysis of results.

Anticipated audience: 1) intro majors course 2) upper level majors course 3) nonmajors course 4) graduate course 5) outreach

Pre-lab Assignments

Reading assignments

1. Overview: Identification for North Atlantic *Porphyra sensu lato* at the genus and species levels using restriction enzymes. (Klein, Brawley and Blouin, *Porphyra* identification Material; <http://www.psaalgae.org/educational-materials/>) Until 2012, *Porphyra* was a single genus with >150 species. However, previous molecular genetic analysis had determined the *Porphyra* and its sister genus *Bangia*, were polyphyletic. Sutherland et al. 2012 began the process revising the taxonomy of the *Porphyra sensu lato*
2. Molecular techniques background
 - a. Gene amplification by the Polymerase Chain Reaction (PCR)
<https://www.dnalc.org/view/15924-Making-many-copies-of-DNA.html>
 - b. Restriction enzymes and Restriction Fragment Length Polymorphism
<https://www.dnalc.org/resources/animations/restriction.html>
 - c. Gel Electrophoresis
<https://www.dnalc.org/resources/animations/gelectrophoresis.htm>
3. Teasdale, B., West, A., Taylor, H. A and Klein A. S. A simple Restriction Fragment Length Polymorphism (RFLP) Assay to discriminate common *Porphyra* (Bangiophyceae, Rhodophyta) taxa from the Northwest Atlantic. J. Appl. Phycol. 38:293-298, 2002.
4. 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.

Pre-lab concept check questions:

ANSWER KEY

1. Why is hard to key out *Porphyra* species using morphological features? “*algal morphology is “plastic” responding to variable ocean and habitat conditions; many species have overlapping diagnostic features” see <http://www.psaalgae.org/educational-materials/>*”
2. What information is needed to design primer pairs to amplify a specific gene from genomic DNA? “*The DNA sequence of the gene, and it’s complementary strand. Computer algorithms are used to select a Forward oligonucleotide primer (14-22 bp) which anneals to the 5’ end of the target region and the reverse primer anneals to the 3’ end of the noncoding sequence. The primer pair should have similar annealing temperatures and should not be able to base pair with each other.*”
3. What DNA sequence is recognized by the restriction enzyme *HinDIII*? “
 - a. 5’A↓AGCTT3’
3’TTCGA↑A5’
 - b. “What do the resulting DNA ends look like? *Over lapping or sticky ends.*
 - b. What DNA sequence is recognized by the restriction enzyme *HaeIII*?
5’GG↓CC3’
3’CC↑GG5’
 - c. What do the resulting DNA ends look like? *Blunt ends*
4. How may a restriction cleavage differentiate similar amplicons from two congeneric different species? *The DNA sequences of the two, congeneric species have diverged at a restriction recognition site, so the enzyme cleaves one but not the other amplicon.*

Sample quiz questions

ANSWER KEY

1. Which of the following are morphological features used to key out blade form of *Porphyra sensu lato* species?
 - a. Moneocious versus dioecious.

- b. Arrangement of reproductive areas of the thallus
 - c. Cell size and shape
 - d. Blade shape
 - e. Blade thickness
2. Why are morphological features frequently inadequate to distinguish between taxa of the family Bangiaceae? *The morphology of intertidal red algae is plastic, sometimes resulting from strength of wave action of the intertidal environment (degree of wave action), showing season variation in the availability of nutrients which influences pigmentation and seasonal variation in the presence or absence of reproductive structure.*
 3. Why is a thermostable Taq DNA polymerase essential to the Polymerase Chain Reaction? *In PCR, the double stranded DNA templates are heated to 93°C to denature the DNA strands, cooled to an temperature (50-60 °C) where primers anneal to either ends of the target region and then warmed to 72°C where the polymerase extends the primer by incorporating nucleotides complementary to the template.; this cycle is repeated 25-35 times. Only thermostable DNA polymerase enzymes, isolated from hot springs bacteria, can withstand multiple rounds of heating and cooling and still stay active.*

Hypothesis

Morphologically similar marine algae often harbor cryptic species.

Post-lab Activities

ANSWER KEY

Quiz questions

1. In which organelle is the ribulose biphosphate carboxylase large subunit gene (rbcL) encoded in *Porphyra*?

Answer: both the small and the large subunits of RuBisCO are encoded in plastid. By contrast, the large subunit of RubisCO is encoded in the plastid of land plants while the small subunit is encoded in the nucleus.

2. Looking at the distribution of restriction fragment sizes for the taxa listed above, what are the commonalities of species in the same genus, compared to different genera?

Answer: The RuBisCO genes of algal in the same genera are more likely to share DNA fragments of the same size, because their genes are more closely related than taxa of the same family, but different genera. The latter genes have diverged further over evolutionary time.

Practical

1. Assuming you had 6000 bp double stranded DNA. If the DNA sequence was random, how often would you expect restriction enzyme *HindIII* to cut (how many fragments) compare to the restriction enzyme *HaeIII*?

- a. # of fragments from *HindIII* cleavage of random 6000 bp fragment $\rightarrow 5000 / 1/4^6 \approx 2$ fragments
- b. # of fragments from *HaeIII* cleavage of random 6000 bp fragment $\rightarrow 5000 / 1/4^4 \approx 24$ fragments

2. Why is a 0.8% agarose gel used to estimate the yield of DNA from the initial extraction while a 2% gel is used to separate restriction fragments?

Electrophoresis applies an electric current where negatively charged DNA moves towards a positive electrode. The pore size of an agarose gel varies with the gel concentration. Genomic DNA is very large (>> megabase pairs in size); in electrophoresis, genomic DNA only slowly penetrates the pores. To

achieve some separation, a very loose gel (0.8 % agarose) allows the genomic DNA to move away from the loading well. The *rbcL* amplicon restriction fragments range from ~60 bp to 1481 bp. To achieve separation of these fragments, the pore size of the agarose gel must be much smaller.

3. Use GenBank sequences and Predict the size and number of restriction fragments produced from *rbcL* amplicons for Northwest Pacific Bangiaceae *Pyropia abbottiae*, *Wildemanina schizophylla*, *Pyropia gardneri*, *Pyropia pulchra* by a common restriction enzymes *HaeIII*, and *HindIII*; if these two restriction enzymes do not discriminate between species, identify one that will **(Bloom scale: Analyzing)**
4. For Graduate Courses: students should identify a moderately conserved gene for *Ulva* species, for which DNA sequences are available in GenBank, and design an assay to distinguish cryptic species (i.e. *Ulva*, *Entromorpha*) in the same genus or family. The assay will use PCR amplification and analysis either DNA sequencing or RFLP analysis) [*Ulva lactuca*, *U. rigida*, *U. compressa*, and *U. pertusa*] **(Bloom scale – creating, evaluating)**

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Pre-Lab Assignments

Name: _____

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Pre-lab concept check questions:

1. Why is hard to key out *Porphyra* species using morphological features?
2. What information is needed to design primer pairs to amplify a specific gene from genomic DNA?
3. What DNA sequence is recognized by the restriction enzyme *HinDIII*?
 - a. What do the resulting DNA ends look like?
 - b. What DNA sequence is recognized by the restriction enzyme *HaeIII*?
 - c. What do the resulting DNA ends look like?
4. How may a restriction cleavage differentiate similar amplicons from two congeneric different species?

Lab Procedure: DNA extraction, gel electrophoresis and set up Polymerase Chain Reaction

GENERAL PRECAUTIONS:

Wear gloves to prevent contamination with nucleases in sweat; change gloves if they get dirty.

Keep tubes on ice.

Keep caps on tubes closed as much as possible, to prevent contamination

Always balance tubes in the microcentrifuge, - make sure tubes diagonally across from each other have the same volume!

The DNA Extraction procedure below is adapted from West 2001, and DNeasy_Plant_Handbook.pdf

- a. DNA Isolation: Prepare sample for grinding
 - i. Freshly collected tissues: rinse with filtered sea water, and blot dry with paper towel. Weigh out ~90 mg from a single thallus. Use a razor blade to chop the tissue into fine pieces, on a microscope slide.
 - ii. Dried tissue (e.g. Main Sea Coast Vegetables' laver.) Weigh out sample of 20 mg. from a single blade. Use a razor blade to chop the tissue into fine pieces, on a microscope slide.
 - iii. Transfer tissues to a small mortar
 - iv. Grind as fine as possible and use a small spatula to transfer tissue to a 2 ml microfuge tube.
- b. Cell Lysis
 - i. Add 400 μ l Buffer AP1, and then 4 μ l RNase A to microfuge tube; cap the tube, and vortex vigorously to break up all clumps.
 - ii. Transfer tubes to 65° heating block for 10 minutes, mix 2-3 times during incubation by gently inverting the tubes.
- c. Precipitation of detergent, proteins and polysaccharides
 - i. Add 130 μ l Buffer P3, mix by inversion
 - ii. Incubate on ice for 5 minutes
 - iii. Centrifuge the lysate for 5 minutes at 14,000 rpm
- d. DNA separation:
 - i. If necessary, cut the ~1 mm. off the bottom of the 1000 μ l ART Pipette tip, to transfer supernatant to QIAshredder Mini spin column (lilac tube)
 - ii. Place column in 2 ml centrifuge tube and spin 2 min. at 14,000 rpm
 - iii. Use a pipette to remove flow thru to a new 2 ml. centrifuge tube, being careful to not disrupt small pellet of cell debris in the bottom of the tube.
 - iv. Estimate the volume recovered, if less than 450 μ l adjust the volume of the reagents added in the next step proportionately.
 - v. Add 1.5 volumes of Buffer AW1 (with Ethanol) and mix by inversion immediately
 - vi. Pipet 650 μ l of solution into DNeasy mini spin column, placed a 2 ml centrifuge tube.
 - vii. Centrifuge 1 min. at 6000 X g (\geq 8000 rpm for most microcentrifuges); discard flow through and reuse the collection tube.
 - viii. Repeat step g with the remaining solution from (e) and repeat centrifuge 1 min. at 6000 X g
 - ix. Place the spin column in a fresh 2 ml. collection tube (supplied), add 500 μ l Buffer AW2 and centrifuge 2 min. at 20,000 X g to dry the membrane; carefully remove the

- Mini spin column without touching the Ethanol flow-through. Discard the collection tube and flow-through.
- x. Transfer the mini-spin column to 1.5 ml microcentrifuge tube; pipet 100 μ l Buffer AE onto the DNeasy membrane.
 - xi. Incubate 5 minutes at room temperature (15-25 $^{\circ}$ C then centrifuge for 1 minute at $\geq 6000 \times g$ (Depending on yield, steps j and k may be repeated, using a second tube to catch the eluate).
- e. Estimate DNA yield:
- i. by loading 5 μ l of each DNA sample mixed with 1.5 μ l of 6X loading buffer into one lane to 0.8% agarose gel; Load 0.5 μ g of 1 kb DNA ladder.
 - ii. Separate at 5 V per cm gel (~45 minutes or until the blue dye moves un electrophoresis till the bromophenol blue band reaches just reaches to the bottom 2/3 of the way down the gel.
 - iii. Move gel to a new plastic tray. Stain in 30 ml of 3X GelGreenTM (in TBE buffer) for 30 minutes, with gentle rocking or swirling.
 - iv. Place on UV Transilluminator³ or Blue Box, and photograph.
 - v. Estimate DNA concentration by comparison to 1 kb ladder
- f. *rhdL* gene + spacer amplification by PCR
- i. Microcentrifuge your DNA extracts briefly (balance the tubes) to bring all solution back to the bottom of the microcentrifuge tube. Put tubes on ice.
 - ii. Prepare PCR master mix:
 - a. Prepare PCR Master Mix in one, 2 ml microcentrifuge tube to have 4 tubes worth for each primer set., vortex briefly to mix, centrifuge, down 2 sec., and store on ice

<u>PCR Reaction equivalents:</u>	<u>one reaction</u>	<u>Master mix</u>
ii. GoTaq Green Master Mix (2X)	25 μ l	100 μ l
iii. Forward Primer (FP 10 μ M)	5 μ l	20 μ l
iv. Reverse Primer (RP 10 μ M)	5 μ l	20 μ l
v. Nuclease-free H ₂ O	10 μ l	40 μ l
vi. DNA template (your sample)	5 μ l	
vii. TOTAL Volume of reaction	50 μ l	180 μ l
 - b. Pipet 45 μ l of Master mix to each 200 μ l tubes in PCR strip.
 - c. Add 5 μ l of your DNA extract to each PCR tube
 - d. Cap the strip; Label the strip top (initials, tube numbers)
 - e. Gently vortex capped strip and spin down before putting into the thermal cycler
 - iii. Start the amplification profile: The amplification profile began with an initial denaturation step of 93 $^{\circ}$ C for 3 min and was followed by 29 cycles of 30 sec at 93 $^{\circ}$ C, 1 min at 45 $^{\circ}$ C, and 1.5 min at 72 $^{\circ}$ C-
 - a. 2-3 hours)/ The instructor will remove your amplifications and store them at 4 $^{\circ}$ C
- 2. Lab 2 confirm PCR amplification, Restriction digestion**
- a. Use gel electrophoresis on a 2% agarose gel in TBE buffer at 5 volts/cm gel to confirm amplification
 - i. Loading 3 μ l of each DNA sample mixed with 0.5 μ l of 6X loading buffer into one lane to 0.8% agarose gel; Load 0.5 μ g of 1 kb ladder.
 - ii. Separate at 5 V per cm gel (~45 minutes or until the blue dye moves 2/3 of the way down the gel.)

³ If students use a transilluminator, either the transilluminator should have a plexiglass cover or the students should wear plexiglass goggles and a face shield

- iii. Move Gel to a new plastic tray Stain in 30 ml of 3X GelGreen (in TBE buffer) for 30 minutes, with
 - iv. Place on UV Transilluminator, and photograph.
 - v. Estimate DNA concentration by comparison to 1 kb ladder
 - vi. *You need at least 0.1µg of DNA per µl to complete the next step*
- b. Restriction digest and sizing DNA fragments
- i. Pipet 25 µl of each PCR product into a fresh, labeled microfuge tube and place on ice.
 - ii. Add 2.5 µl of the 10X restriction enzyme buffer, and mix gently
 - iii. Add 0.5 µl *Hae* III enzyme or *Hind* III to the tube; use a fresh pipet tip for each digest
 - iv. Cap and gently mix the restriction reaction; incubate at 37° C for 10-15 minutes.
 - v. Add 5 µl 6X loading dye to each sample.
 - vi. Load each sample on a 2% agarose gel, with 100 bp DNA standard ladder in one lane.
 - vii. 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.
 - viii. To estimate fragment sizes, generate a standard curve using semilog graph paper. Plot the distance from the well against the log molecular weight of each band in the standard
 - ix. From this graph, you can calculate an equation $\log \text{molecular weight (y)} = -mx + b$, 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.
- c. Identify the “*Porphyra*” unknown from the predicted restriction fragment sizes.

4. Taxon	Hae III fragments	Hind III fragments
<i>Boreophyllum birdiae</i>	237 395 849	1481
<i>Porphyra dioica</i>	99 216 337 382 447	1481
<i>Porphyra linearis</i>	216 482 783	101 1380
<i>Porphyra purpurea</i>	179 1302	101 1380
<i>Porphyra umbilicalis</i>	482 999	101 1380
<i>Pyropia katadae</i>	1481	1481
<i>Pyropia leucosticta</i>	58 179 521 723	1481
<i>Pyropia suborbicula</i>	237 533 711	194 1287
<i>Pyropia yezoensis</i>	58 179 521 723	538 943
<i>Wildemanian amplissima</i>	237 1244	1481
<i>Wildemanian miniata</i>	237 521 723	626 855