Laboratory Protocols for Genotyping *Spartina*

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I. Introduction

The protocols described are those used by the U.C. Davis *Spartina* lab in the laboratory of Professor Donald R. Strong for the purpose of genotyping *Spartina*. The methods were developed by a number of researchers in the laboratory, primarily Drs. Debra Ayres, Christina Sloop and Curtis Daehler. Most of the work is targeted at differentiating native *S. foliosa* from exotic *S. alterniflora* and *S. alterniflora x foliosa* hybrids. There also are RAPD genetic markers that are useful for identifying two other species and hybrids that occur in San Francisco Bay. *S. densiflora* is originally from South America and produces sterile hybrids with *S. foliosa*. *S. anglica* is an allopolyploid hybrid of *S. alterniflora x maritima* that arose in England and was introduced to San Francisco Bay.

Both RAPD and microsatellite markers are useful for diagnosing hybrid *S. alterniflora x foliosa*. RAPDs have the advantage of being relatively fast and inexpensive because they can be scored

with gel electrophoresis. The small differences in base pair length of the microsatellites means they are genotyped on a capillary sequencer, a more costly technology than gel electrophoresis. A two-tiered approach can combine the two methods. First, all samples can be tested with RAPD markers. Next, samples yielding inconclusive or questionable results can be tested with microsatellite markers.

II. DNA Extraction

The U.C. Davis *Spartina* lab has chiefly used two methods for extracting *Spartina* DNA. The first, the kit method, produces DNA that is usable both when analyzing with gel electrophoresis (as with RAPD markers) and for genotyping (for analyzing microsatellite markers). The second DNA extraction method, Proteinase K, produces acceptable results for testing RAPDs with gel electrophoresis, but it may leave behind impurities that interfere with genotyping. Therefore using the kit method is recommended if there is the possibility that a sample may be tested with microsatellites.

A. Kit DNA Extraction Protocol

Use the Qiagen DNAeasy Plant Mini Kit. A detailed instruction pamphlet is included with the kit. Web page:
http://www1.qiagen.com/Products/GenomicDnaStabilizationPurification/DNeasyPlantSystem/DNeasyPlantMiniKit.aspx

B. Proteinase K DNA Extraction Protocol

This protocol was originally based on that of Guidet (1994) but has been substantially modified by Curtis Daehler, Debra Ayres, and other researchers that have worked in Donald Strong's laboratory.

Supply list:

- 1.7 mL eppendorf tubes, labeled with sample number
- 0.5 L liquid nitrogen
- one mortar and pestle per sample
- Kimwipes
- 1 mL extraction buffer stock per sample (see stock solutions)
- 1 mg proteinase K per sample
- dry bath
- hot plate
- 1000 mL beaker
- centrifuge
- 1 mL and 200 µL pipettes and tips
- 2N sodium acetate
- 70% isopropanol
- 100% ethanol
- TE buffer (see stock solutions)

Procedure:

1. Start with fresh, green leaf tissue. Samples can be stored in the refrigerator for a few days prior to processing. They should be free of other living organisms, such as mildew and scale insects (e.g., *Haliaspis spartina*, which look like white dots on the leaves, about ¼" long). Ideally the tissue should be young, such as a green leaf near the top of the tiller; older plant tissue tends to accumulate structural tissues that can interfere with the DNA extraction.
2. Wash plant samples in DI water and dry with Kimwipes.
3. Weigh out 100-120 mg fresh plant tissue. Cut across the leaf into small, 1/8" wide strips. Place in labeled,1.7 mL eppendorf tube.

   Note: At this stage, the tissue can be frozen at -20°C for a few weeks before continuing with the extraction. Upon removing from the freezer, place the tubes on ice until the sample is ground with liquid N in step 5.
4. Dissolve 1 mg of proteinase K per 1 mL of extraction buffer. Make enough for 1 mL of extraction buffer per sample to be extracted.
5. Turn on dry bath and set to 50°C (make sure temp. remains under 56°C)
6. Place plant tissue in mortar; carefully pour small volume liquid N into mortar (enough to fully cover plant sample) and grind plant material into a fine powder.
7. Once liquid N is fully evaporated, add 1 mL of extraction buffer/proteinase K mixture and grind until mixture is a fine slurry.
8. Return sample to labeled tube and place in dry bath for 1 hour to incubate.
9. While waiting for samples in dry bath, boil 800 mL of water in a 1000 mL beaker on a hot plate.
10. After incubation, proteinase K must be denatured by heating. Remove samples from dry bath and turn off hot plate. Place samples in a floating waterbath rack and open the tubes. Float rack in beaker for 5 minutes. When time elapses, remove from water bath, close caps and allow to briefly cool.
11. Centrifuge samples at 5000 Relative Centrifugal Force (RCF) for 10 minutes. The DNA will be suspended in solution.

    Note: RCF = $(1.118 \times 10^{-5})*R*S^2$, where R = radius of rotor in centimeters and S = speed in RPM. The UC Davis laboratory uses an Eppendorf 5415C centrifuge with a 7.3 cm radius, so 5000RCF = 7800 RPM.
12. Pipette 500 µL of the liquid supernatant into fresh, labeled tubes, being careful not to take up any solid debris. Discard pellet and remainder of liquid.
13. Add 10% by volume 2N sodium acetate (50 µL)
14. Add 1 volume 70% isopropanol (500 µL). Invert several times and/or vortex to mix. Place samples in -20°C freezer for at least 1 hour, or overnight. Steps 12 and 13 will precipitate the DNA.
15. Harvest pellet by cold centrifuging at 5000 RCF for 10 minutes.
16. Pour off supernatant and dispose of it. Drain tube upside down on Kimwipe for ~ 1 minute.
17. Dissolve pellet in 100 µL TE buffer. Mix with pipette tip. Change tips between samples.

18. Reprecipitate DNA by adding 10 µL 2N sodium acetate and 250 µL 100% ethanol, in that order. Mix by inversion and/or vortex, then place in -20°C freezer for at least 1 hour or overnight.
19. Harvest pellet as in steps 14 and 15, and dissolve as in step 16.
20. Cold centrifuge at 5000 RCF for 10 minutes.
21. Decant 75 µL of supernatant, being careful not to disturb pellet, into fresh, labeled tubes. Toss pellet and remainder of supernatant.
22. Reprecipitate DNA by adding 7.5 µL 2N sodium acetate and 75 µL 100% ethanol, in that order. Mix by inversion and/or vortex, then place in -20°C freezer for at least 1 hour.
23. Harvest pellet as in steps 14 and 15, and dissolve as in step 16. Centrifuge as in step 19 and decant as in step 20.
24. Find DNA concentration on Nanodrop or spectrophotometer.
25. Dilute DNA sample to 20ng/µL.

    Note: For RAPD analysis, U.C. Davis always diluted DNA using TE buffer. However, excess TE buffer can interfere with genotyping. If samples will be tested with microsatellite primers, diluting with molecular grade water is preferable.
26. Store samples in refrigerator if they will be used within a few weeks. For longer storage, place in freezer at -20°C or below.

III. Analysis with RAPD Markers

Protocol modified from that of Daehler and Strong (1997), Daehler *et al*. (1999), and Ayres *et al.* (1999).

A. PCR

Protocol modified from that described in Blum *et al.* (2004) and Sloop *et al.* (2005).

Supply list:

- Purified DNA (20ng/µL), up to 14 samples
- TE buffer
- 1.7 mL tubes
- 0.6 mL tubes
- Taq: U.C. Davis lab uses Promega GoTaq (5 U/µL)
- Buffer: U.C. Davis uses Promega clear 5x buffer
- $MgCl_2$: U.C. Davis lab uses the $MgCl_2$ that comes with Promega GoTaq (25mM)
- dNTPs (10µM each)
- Molecular grade double-distilled H_2O
- RAPD primers (see Appendix 1A)
- mineral oil
- 96 well PCR plates
- 96 well cooling plate

Note: Prepare a volume of master mix 5-10% greater than required for the total number of PCR assays to be performed, as there will be some pipetting loss.

Procedure:

1. In a 1.7 mL tube, mix reagents to the following proportions
   - 8.154 µL of H_2O per reaction
   - 2.7 µL of 5x buffer per reaction
   - 1.62 µL of $MgCl_2$ (25 mM) per reaction
   - 0.27 µL of dNTPs (10mM each) per reaction
   - .108 µL of TAQ (5u/uL)
2. Vortex to mix
3. Divide mixture into six 0.6 mL tubes in equal proportion. To each submaster mix, add 0.72 µL per reaction of the appropriate RAPD primer.

   Note: A17 requires twice as much primer concentration: add 1.44 ul).
4. Place PCR plate into cooling plate.
5. To each well of the PCR plate, add 13.5 µL of submaster with primer. Group RAPD primers in horizontal rows.
6. Add a drop of mineral oil to each filled well.
7. To each well containing, add 1 µL of DNA, being careful to eject the DNA below the mineral oil. Group each DNA sample in vertical columns.
8. Cover PCR plate with its plastic cover.
9. PCR program:

   1. Denature at 94°C for 1:30
   2. Followed by 40 cycles of:

      94°C for 15 seconds
      42°C for 30 seconds
      72°C for 2 minutes

   3. Hold at 4.0°C.
10. Store in refrigerator until loaded in gel. PCR product can safely be stored in refrigerator for up to a week.

B. Gel Electrophoresis

Supply list:

- Molecular biology-grade agarose (U.C. Davis lab uses GenePure LE)
- 0.5X TBE buffer
- DI H_2O
- PCR product
- 100 BP DNA ladder (see stock solutions)

- 10X Loading dye (see stock solutions)
- Ethidium bromide in solution

Procedure:

1. Prepare a 1.75% gel (i.e. 1.75 grams of agarose per 100 mL TBE buffer).
2. Add .3 µL of loading dye to each PCR well.
3. Load the correct volume of DNA ladder (per the instructions that come with the ladder) to two wells per tier in the gel.
4. Load 13 µL of PCR product to each well in the gel.
5. Run at 120 volts until the loading dye has nearly reached the next tier.
6. Place in ethidium bromide stain for 3 minutes, then move to the DI water bath for 7 – 15 minutes.
7. Photograph under UV light.
8. Score the gel.

The species-specific bands scored at each primer are noted below. The number indicates the base pairs, the name in parentheses indicates in which species the marker occurs.

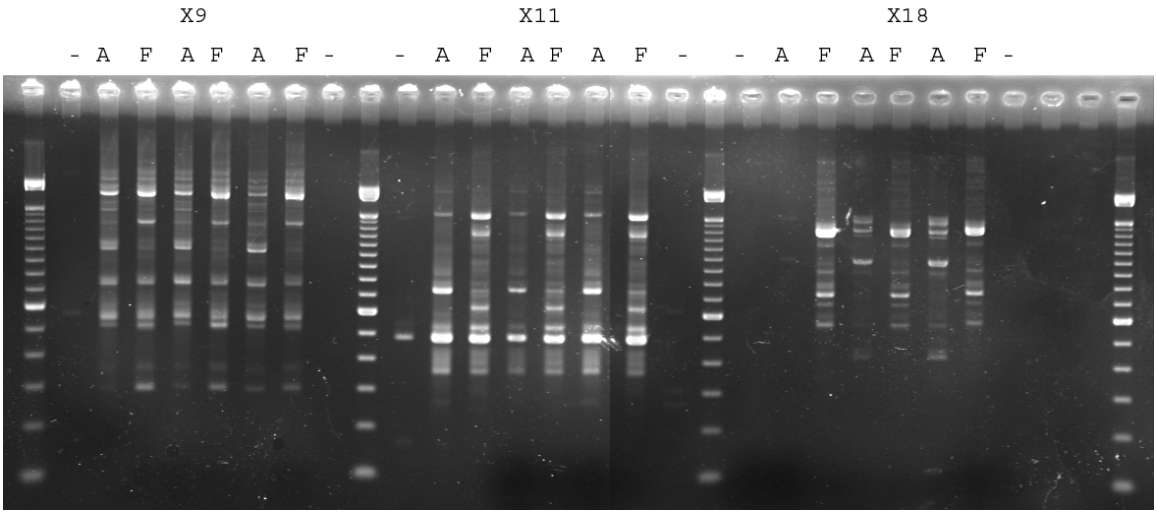
Primer	*Base Pair (species)*
A2	575 (*foliosa*)
A17	725 (*foliosa*)
D5	600 (*alterniflora*), 1100 (*alterniflora*), 550 (*densiflora*), 800 (*densiflora*)
D11	575 (*alterniflora*)
B7 *	550 (*alterniflora*), 650 (*alterniflora*), 800 (*foliosa*/*densiflora*), 700 (*densiflora*), 1200 (*densiflora*)
C10	470 (*alterniflora*)
X9	1000 (*alterniflora*)
X11	750 (*alterniflora*)
X18	950 (*alterniflora*), 750 (*foliosa*)**
B10	750 (*anglica*)
C12	575 (*anglica*)
F10	900 (*anglica*)
G2	1050 (*anglica*)
H7	650 (*anglica*) 750 (*anglica*) 1500 (*anglica*)

* Note: B7 is the trickiest primer to score because *S. foliosa* has a double band at 575 and 625. The alterniflora bands are wide and bright and can occur either at 550, 650, or both. An individual *S. alterniflora* plant could have either band or both (but always has at least one).

** X18 also has a species-specific band at 450 for *S. alterniflora*, but Laura Feinstein of U.C. Davis had difficulty getting this band to show up clearly in her gels.

Image of the X9, X11, and X18 tests:

"-" indicates the negative controls, "A" indicates alterniflora, and "F" indicates foliosa. The rest of the lanes are ladder or are empty.



IV. Analysis with Microsatellite Markers

A. PCR

Supply list:

For each reaction, the following amounts are added to yield a total volume of 14 uL:

- 5 µL DNA template at 20 ng/µL
- 2 µL 10 x buffer
- 0.5 µL dNTPs (10 µM)
- 1.5 µL forward labeled primer (10 µM)
- 1.5 µL reverse primer (10 µM)
- 0.03 µL TAQ DNA polymerase (5 U/µL)
- $MgCl_2$, volume depending on optimal concentration for primer (see Appendix 1B)
- Add molecular grade double distilled water to make a total volume of 14 µL

Procedure:

1. To make master mix for a 96 well reaction, multiply all reactants by 100 and add to a 1.7 mL Eppendorf tube stored on ice.
2. Vortex master mix for a few seconds, then centrifuge at ~16000 RCF for 3-5 seconds.
3. Aliquot 9 µL of Master Mix into each tube of a 96 well plate stored on a cold block.
3. Add 5 µL of DNA (20ng/µL) to each tube.
4. Cover each plate with a sealing mat.

5. Run the PCR program appropriate for the primer.

PCR program:
1. Denature at 94°C for 2 minutes

2. 29 cycles of:

94°C for 0:45 seconds
T_a for 45 seconds (see Appendix 1B)
72°C for 1 minute

3. Extension phase, 72°C for 6 minutes

4. Hold at 4°C

6. Store PCR plate at -20°C.

B. Genotyping

1. Quantify the DNA concentration in the PCR product. This can be done by running 2.5 µL of the PCR product on a 2% agarose gel with a marker of known concentration, or by using a 96-well plate reader.
2. Dilute the product to the concentration required by the sequencing instrument using double-distilled water.

   Note: For the Genomics Facility at UC Davis, PCR products ranging from 100-200 base pairs in length should be at a concentration of 0.2-0.6 ng/µL. PCR products from 200-500 base pairs in length should be 0.6-2.0 ng/µL. Primers SPAR. 20, 8, 2, and 18 yield products from 100-200 base pairs long, while all other primers give products from 200-500 base pairs long.
3. Transfer 5 µL of product from each well of the PCR plate to the corresponding well in the multiplex plate. Up to 5 sets of products labeled with different fluorescent dyes and/or of differing base pair lengths can be added to a plate. Recommended multiplex groups are given in Appendix 1.
4. After all products have been added to the multiplexed plate, seal with USA scientific sealing film and label. Store at -20°C.
5. Genotype the products.

   Note: The facility at UC Davis uses an ABI 3730 XL capillary sequencer from Applied Biosystems.
6. Alleles characterizing *S. foliosa* and hybrids are listed in Appendix 1B. The presence of any one allele that is not listed in column four, "*S. foliosa* alleles," is evidence that the sample is a hybrid plant.

V. Literature Cited

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Appendix 1 - Primers

A. RAPD Primers

A single RAPD primer serves as both the forward and reverse primer.

We use standard, salt-free primers ordered from Invitrogen and dilute with TE buffer to a concentration of .004 nM/μL.

Standard set for diagnosing *S. alterniflora, S. foliosa, S. alterniflora x foliosa, and S. densiflora* (these six primers were used for San Francisco Estuary *Spartina* assessments between 2001 and 2008):

A2	TGCCGAGCTG
A17	GACCGCTTGT
D5	TGAGCGGACA
D11	AGCGCCATTG
B7	GGTGACGCAG
C10	TGTCTGGGTG

Additional primers for diagnosing *S. alterniflora, S. foliosa, and S. alterniflora x foliosa* (these three primers were refined by Laura Feinstein in 2009):

X9	GGTCTGGTTG
X11	GGAGCCTCAG
X18	GACTAGGTGG

Additional primers for diagnosing *S. anglica*:

B10	CTGCTGGGAC
C12	TGTCATCCCC
F10	GGAAGCTTGG
G2	GGCACTGAGG
H7	CTGCATCGTG

A note on the *S. anglica* primers: these primers were developed by Debra Ayres to distinguish *S. maritima*, *S. alterniflora* and *S. anglica*. However, these primers have never been tested with *S. foliosa* and so it is unknown what bands an *S. foliosa* or and *S. alterniflora x foliosa* hybrid would generate with these primers.

B. Microsatellite Primers

The primers are listed in approximate order of their usefulness in diagnosing hybrids as a function of how often they successfully amplify the target product and the population frequency of hybrid-specific alleles. The last column in the table, $MgCl_2$ (µL) gives the volume to add of $MgCl_2$ when using 2 µL/reaction of Qiagen 10x PCR buffer, which already contains 15mM $MgCl_2$.

Primer ID	*Forward sequence*	*Reverse sequence*	*S. foliosa alleles*	*Alleles occurring only in hybrids*	*T_a* (°C)*	*$MgCl_2$ (mM)*	*$MgCl_2$ (µL)*
Spar.15	ATTTGCTGCTTTTGGTAGAC	GTAGAACAATGGAAGAATGC	266	269, 271, 273, 275, 279, 285, 296	51	2.83	0.39
Spar.26	TTCAACTGGCGTAGTGATTCC	AACATTTCCGACTGGTAGAGC	263	275, 277, 281, 283, 285, 286, 291	58	3.17	0.58
Spar.20	ACCGTGCCTCAGCTACTG	GGTGTTTCCTCGCATAGATC	171	173, 175, 177, 179, 181	52	2.17	0.02
Spar.23	GGGAAGTGAAATCTGGTTGC	GCTTGCTTGTCTCAGTCC	262, 264, 266, 268	248, 250, 274	55	2.17	0.02
Spar.25	CGGTAGAGACGGAGTTGTGG	GCTTGGGAGATGAGACTGGAC	245, 249	253	69	3.00	0.48
Spar.08	CTAAGGTCCCAAACGACGAC	GCGACGAGCGAGGATTTAC	193	180, 188	58	2.50	0.20
Spar.28	CACCGTTCAATCACAGTT	GGAAGCAGGAGGGGTTGG	416	419,476	59	2.00	0.00
Spar.02	GAAGGACGAGTCTCATTTGG	GGCTGCCCCTGTTTCACG	193	201, 213	56	2.50	0.20
Spar.27	CATCAAAAGCAAGAGGA	GACACCAACGGAACTG	314	304, 309, 321, 323, 328, 331	50	2.17	0.02
Spar.09	GTGGCCTAGCCTATCGACCT	TGAATGGAAAGGGGAAATGA	279, 285, 294	273, 277, 292, 296	58	2.50	0.20

* T_a = Annealing temperature

Groups suitable for multiplexing together (no more than two overlapping size ranges):

Plate 1.	Plate 2.
Spar.15	Spar.23
Spar.26	Spar.28
Spar.20	Spar.02
Spar.25	Spar.27
Spar.08	Spar.09

Appendix 2 – Stock Solutions

A. DNA Extraction Buffer (10mM Tris, 0.45M ETDA, 1% lauryl sarcosine)

Note: When working with NaOH or HCL, wear eye protection and gloves.

1. Place in a 500 mL fleaker:
   - 6 gm Tris
   - 83.7 gm EDTA
   - 5 gm N-lauroyl sarcosine
2. Add DI water to 400 mL and place on stir plate until the mixture is clear.
3. Make saturated NaOH (place 10-15 NaOH pellets in flask, add 50 mL DI water, stir until dissolved, add more NaOH pellets and stir until dissolved until some undissolved pellets remain).
4. Add 20 mL saturated NaOH.
5. Adjust pH to 8.0 with NaOH and/or HCL.
6. Measure up to 500 mL in a graduated cylinder.
7. Split into 2 fleakers and autoclave.

B. TE Buffer (10mM Tris, 1mM EDTA) ph 7.8

1. Place in a 500 mL fleaker
   - 6 g Tris
   - 0.19 g EDTA
2. Add 500 mL DI water, stir until dissolved
3. Divide into two 250 mL flasks and autoclave.

Note 1: Wear gloves when handling plant matter or DNA to avoid contaminating the samples.

Note 2: You will need to wear safety glasses, gloves and closed toed shoes to work with liquid N.

C. 10X Loading Dye

1. Mix in small beaker
   - 0.042 g Bromine Blue
   - 6.6 g sucrose
   - 10 mL TE buffer
2. Bottle and remove 1.5 mL into 1.5 mL tube for use.

D. 100 BP DNA ladder

1. Mix in 1.5 mL tube

- 50 µL 100 BP Invitrogen ladder (Cat no. 15628-019)
- 160 µL 5X Promega buffer from the GoTaq kit
- 80 µL 10X loading dye (see above)
- 510 µL DI H_2O

2. Divide into five aliquots of 160 µL in .7 mL tubes. Use one of these tubes at a time (it can remain on the workbench) and freeze the rest until needed.

E. 10X TBE buffer

1. Place in 1 L fleaker
   - 108 g Tris
   - 55 g boric acid
   - 8.3 g EDTA
2. Add DI H_2O to bring up to 1 L. Add stir bar and place on stir plate until clear (should take 20 minutes or more)
3. Cap with aluminum foil.

F. DNTPs

The directions below are for making a dNTP mixture of 10 mM per dNTP. The volumes given are based on Promega dNTPs which come at 100 mM concentration.

1. Combine in a 1.5 µL tube
   - 50 µL Promega dATP
   - 50 µL Promega dTTP
   - 50 µL Promega dGTP
   - 50 µL Promega dCTP
   - 300 µL molecular grade ddH_2O
   2. Mix by vortexing, split into 5 100 µL batches in .7 mL tubes, and freeze.