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Title: Fish Speciation by Isoelectric Focusing		
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Contents

A. INTRODUCTION

1. Theory

   Sarcoplasmic proteins are extracted from catfish and analyzed by isoelectric focusing. These sarcoplasmic protein profiles are species-specific and reproducible. By using the isoelectric points (pIs) and gel electrophoresis for separation, an individual class can be determined from the specific banding patterns its proteins create. Samples are compared to a known standard for identification.

2. Applicability

   This method is suitable for the species determination of fish.

B. EQUIPMENT

Note: Equivalent apparatus or instrumentation may be substituted unless specified for any of the following.

1. Apparatus
   a. Vortex mixer - with multi-sample starter set, Cat. No. 0221573, Fisher Scientific.
   b. Micro centrifuge - Model 235 B, Fisher Scientific.
   c. Biomax 5 kD cutoff filters - Cat. No. 42404, Fisher Scientific.
   d. Micro centrifuge tubes, 1.5 mL - Cat. No. 20901-646, VWR.
   e. Micro centrifuge tubes, 0.5 mL - Cat. No. 20901-645, VWR.
   f. Transfer pipettes - Cat. No. 13-711-7, Fisher Scientific.
   g. Transfer pipettes - Cat. No.231, Samco Scientific Corp.
   h. Scalpel - Cat. No. 139095, Lab Depot.
   i. Trays - plastic.
   j. Shaker plate - Thermolyne BIG Bill.
   k. Ultra low freezer - -70 °C freezer or another freezer that does not undergo freeze-thaw cycles and maintains at least -20 ºC.

2. Instrumentation
   a. Isoelectric focusing unit with - Criterion Cell, Cat. No. 165-6019, Bio-Rad, equipped with:
      i. proper electrode geometry.
      ii. power supply; constant-power type capable of maintaining constant

power of ≥ 1- 30 watts.

Note: The power supply produces dangerously high voltages and caution should be taken when the power supply is on. Safety contacts should never be circumvented.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents or solutions may be substituted unless specified for any of the following.

1. Reagents

   a. 1 M NaOH - electrophoresis grade, Cat. No. SS266-1, Fisher Scientific.

   b. 1 M H_3PO_4 - Cat. No. S8282, Sigma Aldrich.

   c. Isopropanol - Cat. No. 6351-8601-7-40, KSE Scientific.

   d. 85% Phosphoric Acid - Cat. No. A260-500, Fisher Scientific.

   e. Sulfosalicyclic acid - Cat. No. BP177-500, Fisher Scientific.

   f. Trichloroacetic acid - Cat. No. A322-500, Fisher Scientific.

   g. Methanol - Cat. No. A452-4, Fisher Scientific.

   h. De-ionized water (DI water) - Millipore or molecular grade.

   i. Acid Violet 17 - Cat. No. 210579-50G, Sigma Aldrich.

   j. IEF 10x Anode Buffer - Cat. No. 161-0761, Bio-Rad.

   k. IEF 10xCathode Buffer - Cat. No. 161-0762, Bio-Rad.

   l. Criterion IEF gel pH 5-8 - Cat. No. 345-0076, Bio-Rad.

   m. Glycerol - Cat. No. BP229-1, Fisher Scientific.

   n. Acetic Acid, glacial - Cat. No. JT9522-33, VWR.

2. Solutions

   Note: Trichloroacetic acid should be weighed and reagents should be prepared in a fume hood.

a. Fixing Solution:

Combine 40 g of sulfosalicyclic acid, 125 g of trichloroacetic acid and 300 mL of methanol to 700 mL DI water in a bottle. Solution is stable for six months.

b. 0.1 M NaOH:

To 45 mL of DI water add 5 mL of 1 M NaOH in a bottle and mix thoroughly.

c. 3% Phosphoric Acid (De-staining Solution):

To approximately 1500 mL of DI water in a 2 L volumetric flask, add 70 mL of 85% phosphoric acid and bring to volume with DI water.

d. 11% Phosphoric Acid:

To 157 mL of DI water add 23 mL of 85% phosphoric acid in a bottle and mix thoroughly. Make fresh each day.

e. 1% Acid Violet 17 stock solution:

Add 1 g of Acid Violet 17 to a 100 mL volumetric flask and bring to volume with DI water. Heat to 55 ± 5 °C while stirring until dissolved.

f. 0.1% Acid Violet 17 staining solution:

Add 20 mL of 1% Acid Violet 17 Stock Solution to 180 mL of 11% phosphoric acid into a graduated cylinder.

g. Preserving Solution:

Add 50 mL of glacial acetic acid, 25 mL of methanol, and 50 mL of glycerol to 375 mL of deionized water in a bottle. Mix thoroughly. Solution is stable for six months.

h. Sample Buffer:

Add 10 mL of glycerol to 10 mL of double deionized water in a bottle. Mix thoroughly.

i. 1x Anode Buffer:

Add 40 mL of 10x Anode Buffer to 360 mL of double deionized water in a bottle. Mix thoroughly. Refrigerate solution for storage and may be re-used up to 4 times before discarding.

j. 1x Cathode Buffer:

Add 6 mL of 10x Cathode Buffer to 54 mL of double deionized water in a bottle. Mix thoroughly. Refrigerate solution for storage and must be discarded after every use.

D. STANDARDS

1. Source - Authenticated specimens of a channel catfish (*Ictalurus punctatus*), blue catfish (*Ictalurus furcatus*), and channel x blue hybrid provided by the Georgia Department of Natural Resources, University of Georgia, or an equivalent source.
   a. Authenticated catfish samples: Must have a certificate of authentication stating the species of catfish.
   b. pI standards, pH 3 - 9 or another of appropriate range – Cat. No. 161-0310, Bio-Rad.
2. Storage and Stability
   a. Storage:
      i. Store all catfish standards in -70 ºC freezer or in a -20 ºC freezer that does not go through freeze/thaw cycles. Keep standards in an air-tight container/wrap, preferably vacuumed-sealed to prevent freezer burn.
      ii. Store pI standard per manufacturer requirements.
   b. Stability:
      i. Catfish standards can be used until the specific banding patterns are not obvious or absent. When appropriately stored, standards are stable indefinitely.
      ii. pI standards may be used up to their expiration dates.

E. SAMPLE PREPARATION

1. Sample Receipt
   a. Samples are usually received as whole fish or in filets.
      i. Whole fish: Filet the fish using a sterile knife. Store in an air-tight container at -20 ºC or below until time of extraction.
      ii. Filet: Store in an air-tight container at -20 ºC or below until time of extraction.

F. ANALYTICAL PROCEDURE

Note: Authenticated fish samples are used for comparison (e.g. to confirm species or to rule out a species), and the availability of the appropriate authenticated sample must be determined prior to performing the analysis.

1. Extraction
   a. All test samples should be analyzed along with the appropriate standards which may include blue, channel, and hybrid catfish standards. Keep the samples and

standards as cold as possible throughout the process by working on ice when possible and minimizing the time samples are at room temperature. Return standards and samples to the freezer immediately after obtaining the necessary portion.

b. With a sterile razor blade, cut from the meaty portion of the fillet about 0.6 g of tissue (0.3 g - 1.0 g) and place into a 1.5 mL microcentrifuge tube.

c. To each tube add approximately 0.5 mL - 1.0 mL until the tube is full of cold deionized water. Tissue must be covered completely by water. Vortex the sample vigorously about 1 minute.

d. Let the samples sit on ice for 30 ± 10 minutes.

e. Centrifuge using the microcentrifuge for 2 minutes at 10,000 rpm, or until the sample is packed in the bottom of the tube.

f. Transfer the supernatant to a 5 kD cut off filter and centrifuge for 2 minute ± 30 seconds at 10,000 rpm.

g. Transfer the top portion to a clean, cold microcentrifuge tube.

Note: Occasionally it may be necessary to add a small amount of cold deionized water to the top part of the cutoff filter to solubilize the proteins (i.e. if insufficient supernatant is remaining above the filter).

h. Refrigerate extracts until ready to load on the gel. This preparation must be used by the next day.

i. Add 40 µL ± 1 µL of the extracted liquid to 5 µL ± 1 µL of sample buffer in a separate microcentrifuge tube; mix well. Other volumes are acceptable as long as this dilution factor is maintained.

2. Gel preparation and analysis

a. Open up the individually packaged criterion gel, remove the comb and gently rinse the wells with molecular grade water.

b. Remove the tape across the bottom of the gel by gently pulling on the tab and insert the gel into the Criterion cell with the upper chamber facing toward the middle of the cell.

c. Pour 60 mL ± 5 mL of cathode buffer in the upper chamber and 400 mL ± 10 mL of the anode buffer in the bottom chamber.

d. Load 13 µL ± 5 µL of diluted sample into well on gel. Load pI marker on gel bracketing samples. Place lid on the tank making sure the color coded banana plugs and jacks are aligned.

e. Turn on the power supply and run the power program using appropriate settings. The following settings are an example of a program run:

i. 100V, 25mA, 10W; 30 minutes

ii. 175V, 25mA. 10W; 30 minutes

iii. 250V, 25mA, 10W; 50 minutes

iv. 300V, 25mA, 10W; 50 minutes

3. Developing the Gel

a. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.

b. Remove the lid from the tank, and remove the Criterion gel(s) from the cell. Pour off and discard the cathode buffer. Anode buffer may be retained and reused for up to 4 ± 1 runs; buffer must be refrigerated.

c. Invert the cassette and place the integral buffer chamber over the cassette-opening tool built into the Criterion cell lid. Firmly press down on the cassette to crack the welds open on both sides of the cassette. The cassette will split open approximately 1/3 of the way. Pull the two halves apart to completely expose the gel.

d. Remove gel by floating it into the fixing solution.

e. After 30 minutes pour off the fixing solution into a waste container and cover the gel with deionized water. Shake gently.

f. Pour off the deionized water into waste container and cover the gel with 3% phosphoric acid. Shake gently.

g. Pour off the 3% phosphoric acid into a waste container and cover the gel with the 0.1% Acid Violet 17 Staining Solution and gently shake for 30 minutes ± 5 minutes.

h. Pour off the staining solution into a waste container and de-stain with 3% phosphoric acid until the background is clear.

i. Pour off the 3% phosphoric acid into a waste container and cover the gel with preserving solution and shake for 10 ± 5 minutes. Pour off preserving solution into a waste container.

j. Place gel into a clear resealable plastic bag.

G. RESULTS AND RECORD

1. Take a photograph of the gel, and save the image file to the designated folder set up in the local intranet. Place the gel in a refrigerator and put hardcopies of the image with each data packet.

2. Compare the protein bands of the sample to the protein bands of the authenticated standard. Due to variations of the gels on different runs, comparison should only be made between the standards and samples run on the same gel. The result will be considered a positive identification if the banding patterns match without significant

difference. The absence of 1 or 2 bands from the test sample is usually not considered significant. The intensity should not be considered. An example of banding patterns is shown below in Figure 1.

Note: Additional reference IEF banding patterns for fish species can be found at the FDA/CFSAN Regulatory Fish Encyclopedia website: http://www.cfsan.fda.gov/~frf/rfe0.html

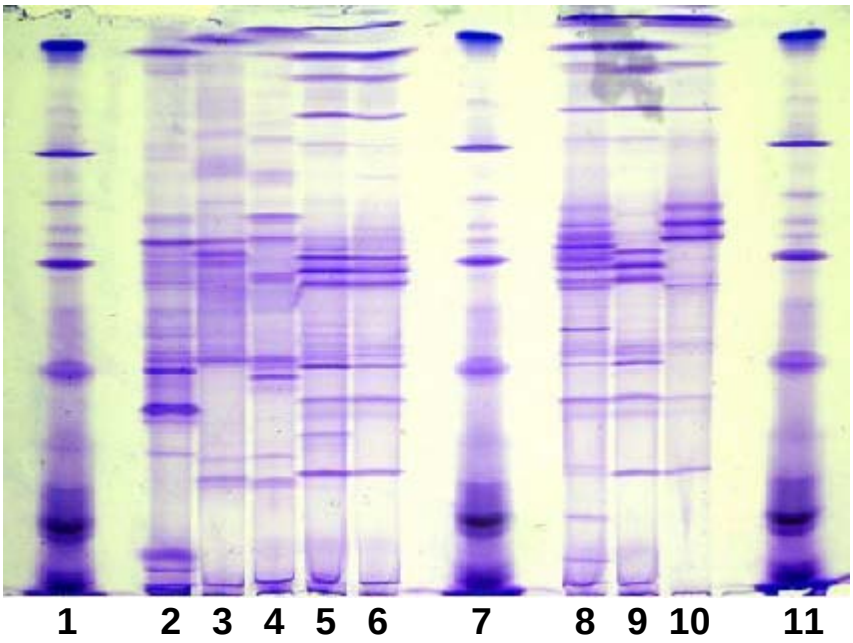

Figure 1. IEF Banding Patterns for fish.
1, 7, 11. pI markers; 2. Gag Grouper; 3. Wavy Lined Grouper; 4. Camoflage Grouper; 5. Channel Catfish; 6. Channel Catfish; 8. Certified Hybrid Catfish (Blue x Channel); 9. Certified Channel Catfish; 10. Certified Blue Catfish.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - laboratory coat, eye protection, and gloves.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic Solvents (Methanol, Isopropanol)	Flammable. Vapors are corrosive to skin, eye, and respiratory system	Avoid contact or prolonged exposure to vapors. Work in fume hood. Keep away from flame or heat.

Acid and Bases (H_3PO_4, NaOH)	Corrosive. Contact with liquids can result in burns and severe skin, eye and respiratory irritation.	Prepare solutions using these reagents with care in a well-ventilated area such as a fume hood. Wear protective eyewear, gloves and clothing when handling.
Trichloroacetic acid	Corrosive, severe burns to mucous membranes	Wear protective clothing, use in well ventilated area
Polyacrylamide gels	Unpolymerized-suspected carcinogen	Wear protective clothing, use in well ventilated area
Sulfosalicylic acid	Slight Irritation of skin	Wear protective clothing
Acid violet 17	Irritation, skin contact may discolor skin due to pigment	Wear protective clothing

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
All acids and bases - including Trichloroacetic acid and Sulfosalicylic acid (see above)	See Hazards, above	Neutralize solutions to meet local, state, and federal guidelines.
Organic Solvents (see above)	See Hazards, above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state and federal regulations.
Acid Violet 17	See Hazards, above	Dispose of according to local, state, and federal guidelines.
Polyacrylamide gels	See Hazards, above	Dispose of according to local, state, and federal guidelines.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. A developed gel must meet the following criteria:
      i. Banding pattern specified for the pI standards must be present.
      ii. Banding pattern of authentic fish standards are within expected pI range.

2. Critical Control Points and Specifications

Step	*Record*	*Acceptable Control*
E.1.	Storage temperature	Freezer (-70º C) or -20 ºC without freeze/thaw cycle.
E.2.a.	Fish handling temperature	Keep fish samples on ice as much as possible.

3. Readiness To Perform
   a. Familiarization
      i. Phase I: Not applicable.
      ii. Phase II: Fish samples that will include Ictalurids and fish from other classes and families. Complete 3 sets over at least 3 different days. A set must include:
         (a) pI Markers.
         (b) Catfish standards (blue, channel and hybrid).
         (c) Six additional fish samples. Three of these six must be known catfish samples, while the other three must be known non-catfish samples.
      iii. Phase III: Check samples for analyst accreditation.
         (a) A minimum of 8 unknown fish samples to include fish from both the Ictaluridae family and fish from other families. At least three unknowns must be from the Ictaluridae family.
         (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM) and Supervisor.
         (c) Authorization from QAM and Supervisor is required to commence official analysis.

b. Acceptability criteria.

Refer to section I.1. above.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Once per week per analyst, when analyses are performed.

ii. Records are to be maintained for review.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: fish muscle.

b. Condition upon receipt: frozen.

c. Sample storage:

i. Time: One year.

ii. Condition: Frozen.

6. Sample Set

Each sample set must contain:

a. pI Standards.

b. Authentic fish standards.

c. Test samples to be analyzed.

7. Sensitivity

Not applicable.

J. WORKSHEET

(Reserved)

K. APPENDIX

1. References
   a. AOAC Official Method 980.10 - Identification of Fish Species.
   b. SOP No. FL Method 595 Fish Species Identification by the Analysis of Water Soluble Sarcoplasmic Proteins using Isoelectric Focusing Gel Electrophoresis (Florida Department of Agriculture and Consumer Services, Bureau of Food Laboratories).

L. APPROVALS AND AUTHORITIES

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Division.