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Title: Fish Speciation by PCR/Sequencing of Mitochondrial Cytochrome Oxidase I (COI) Gene		
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Contents

A. INTRODUCTION

1. Background

All catfishes are members of the order Siluriformes, which contains 36 families and over three thousand species. It is important to be able to distinguish between properly and improperly labeled fish. The application of DNA barcoding with the mitochondrial cytochrome oxidase I gene (COI) for species identification lends itself to this task. This prevents both the fraudulent mislabeling of products and the risk of potential health hazards associated with some specific species. Catfish production in the United States consists predominantly of two farmed species, channel (*Ictalurus punctatus*) and blue (*I. furcatus*) catfish, as well as hybrids of these two species.

2. Summary of Procedure

This method focuses upon the DNA sequence of the mitochondrial cytochrome oxidase I (COI) gene. DNA from muscle tissue is isolated and the COI gene is amplified using the polymerase chain reaction (PCR) and sequenced by capillary electrophoresis. The subsequent DNA sequence is compared with that of a known (i.e. vouchered and certified) specimen and used to identify that sample.

3. Applicability

This method is suitable to verify the identity of siluriformes unprocessed muscle tissue by comparing the sequence of its mitochondrial CO1 gene to authenticated sequences.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Vortex Genie 2 - Model No. G560, Scientific Industries.

b. Swing-Out Bucket Rotor - Cat. No. A-2-DWP, Eppendorf.

c. Fixed-Angle Microcentrifuge Rotor - Cat. No. F-45-30-11, Eppendorf.

d. Refrigerated Centrifuge - Model No. 5804R, Eppendorf.

e. Micropipettors - volume capacities of between 1and 1000 µL, various catalog nos., Rainin.

f. Multichannel Micropipettors - appropriate tips for use with 96-well plates, Rainin.

g. Self-Sealing Barrier Pipette Tips - sterile, same Volumes as f. above.

h. Microcentrifuge Tubes - 1.5 mL, sterile, Cat. No. 20170-038, VWR.

i. Microcentrifuge Tubes - 0.5 mL, Cat No. 80087-084, VWR.

j. PCR Tubes - sterile, 0.2 mL, Cat. No. 20170-012, VWR.

k. Syringe filters - sterile, 0.2 mL, Cat. No. 28155-477, VWR.

l. Racks for Microcentrifuge and PCR Tubes - Cat. No 20901-675, VWR.

m. Disposable Scalpels - sterile, Cat. No. 21919-664, VWR.

n. 30" PCR Workstation - Cat. No. P-030-02, C.B.S. Scientific.

o. Precast agarose gels - Cat. No. 6018-02, Invitrogen.

p. Shaking Water Bath, temperature controlled capable of maintaining 50 - 60 ºC inclusive - Cat. No. SW-23, Julabo.

q. Sample Microtiter Plates - Cat. No. 4306737, Applied BioSystems.

r. Aluminum Sealing Film - Cat. No. 60941-076, VWR.

s. Clear Sealing Film - Cat. No. 10011-119 VWR.

t. Heat-sealing Film, - Cat. No. 82018-846, VWR.

2. Instrumentation

a. Thermal Cycler - Model No. C-1000, Bio-Rad.

b. Gel Documentation System - Model No. Gel Doc XR, Bio-Rad.

c. Genetic Analyzer - Model No. 3130xL or 3500xL, Applied BioSystems.

d. Electrophoresis power supply - Cat. No. G6465, Invitrogen.

e. Minicentrifuge - Cat. No. 93000-196, VWR.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted.

1. Reagents

a. Sterile Water - NANOpure

b. DNeasy™ Blood and Tissue Kit - Cat. No. 69504, Qiagen.

The kit contains the following:

i. DNeasy™ mini spin columns in 2-mL microcentrifuge collection tubes

ii. Buffers ATL, AL, *AW1 (concentrate), *AW2 (concentrate), and AE

iii. Proteinase K

iv. Additional 2-mL microcentrifuge collection tubes

** Note: Ethanol must be added to these two buffers prior to use, according to manufacturer's specifications.*

c. Absolute ethanol, Cat. No. 51976, Sigma Aldrich.

d. D-(+)-Trehalose dehydrate - Cat. No. T9531, Sigma Aldrich.

e. 10 mM Deoxynucleotide (dNTP) Solution Mix - Cat. No. N0447L, New England Biolabs.

f. COI Oligonucleotide Primers – Primer A: FishCO1LBC_ts (5'-CAC GAC GTT GTA AAA CGA CTC AAC YAA TCA YAA AGA TAT YGG CAC -3' and Primer B: FishCO1HBC_ts (5'-GGA TAA CAA TTT CAC ACA GGA CTT CYG GGT GRC CRA ARA ATC A-3') as described by Handy et al 2011 and custom-made by Integrated DNA Technologies

g. Sequencing Oligonucleotide Primers - 10 µM each, M13F (-29) (5'- CAC GAC GTT GTA AAA CGA C-3') and M13R (5'- GGA TAA CAA TTT CAC ACA GG-3') available from Integrated DNA Technologies.

h. Platinum Taq DNA Polymerase Kit - Cat. No. 10966-034, Invitrogen.

The kit contains the following:

i. Platimum® Taq Polymerase

ii. 10X PCR Buffer, Minus Mg

iii. 50 mM Magnesium Chloride

i. 100 bp DNA Ladder - Cat. No. 15625-019, Invitrogen.

j. ExoSAP-IT® - Cat. No. 78200, Affymetrix.

k. BigDye® Terminator v3.1 Cycle Sequencing Kit - Cat. No. 4337457,Applied Biosystems.

l. Performa®-DTR Gel Filtration Cartridges – Cat. No. 42453, EdgeBio.

m. Performa®-DTR V3 96-Well Short Plates – Cat. No. 63387, EdgeBio.

n. POP-7 Polymer for 3130 DNA analyzers – Cat. No. 4352759, AppliedBiosystems.

o. 5X Sequencing Buffer - Cat. No. 4336697, Applied Biosystems.

p. Capillary Array for either the 3130xL or 3500xL Genetic Analyzer - Cat. 4315931 or Applied Biosystems.

q. Hi-Di™ Formamide - Cat. No. 4311320, Applied Biosystems.

2. Solutions

a. 50 µM COI Primer Stock Mixes :

Re-suspend each primer (from the pellet and still in its original tube) in

approximately 500 µL of sterile water. The exact volume is dependent upon original manufacturer concentration. Store primers for up to eighteen months at ≤ -20 ºC while not in use.

b. 10 µM COI Primer Working Solutions:

Pipette 20 µL of primer stocks into separate 0.5 mL microcentrifuge tube and dilute to 100 µL with sterile water. Store both solutions for up to eighteen months at ≤ -20ºC while not in use.

c. 10% Trehalose Solution:

Dissolve 1 g of trehalose into a 10 mL volumetric flask, bring to volume with sterile water, and filter-sterilize. Using a micropipettor, dispense solution into multiple 1.5 mL microcentrifuge tubes and store them up to one year at ≤ -20ºC while not in use.

d. 10 µM Sequencing Primers:

Re-suspend primers (from the pellet and still in their original tubes) in approximately 200 µL of sterile water. The exact volume is dependent upon original manufacturer concentration. Store primers for up to eighteen months at ≤ -20ºC while not in use.

e. COI PCR Master Mix

i. Thaw Primers A and B, Sterile H_2O, 10% trehalose solution, Buffer, $MgCl_2$, dNTP mix, and Taq polymerase in ice bucket. Invert or flick tubes several times to mix and centrifuge for 5 - 10 sec in mini-centrifuge.

ii. Using the table below, prepare the appropriate volume of master mix for PCR amplification of cytochrome oxidase I gene in a microcentrifuge tube. Mix by pipetting up and down several times or by vortexing gently. Spin briefly in mini-centrifuge. Either use master mix immediately or store in freezer at ≤ -10° C for up to six months.

Reagent Name	Per 1 Reaction (µL)	Per 100 Reactions (µL)
10% trehalose	6.25	625
Sterile water	2.0	200
10X Buffer	1.25	125
50 mM MgCl2	0.625	62.5
10 µM Primer A	0.125	12.5
10 µM Primer B	0.125	12.5
10 mM dNTP mix	0.062	6.2
Taq polymerase (5 U/mL)	0.06	6.0
Total	10.5	1050

f. Sequencing Reaction Master Mix

i. Thaw tubes of trehalose, sterile water, primers M13F and M13R, and sequencing buffer. When they are completely thawed, invert them several times to mix the reagents and centrifuge for 5 - 10 seconds.

Note: BigDye™ is light and temperature sensitive and should remain covered and in the freezer except when in use.

ii. Using the table below, prepare sequencing master mix using either Primer M13F **or** Primer M13R. Either use master mix immediately or store in freezer at ≤ -10° C for up to six months.

Reagent Name	Per 1 Reaction (µL)	Per 100 Reactions (µL)
10% Trehalose	5.0	500
Sterile water	0.875	87.5
10 µM M13F **OR** M13R	1.0	100
5X Sequencing Buffer	1.875	187.5
Big Dye™	0.25	25
Total	9.0	900

D. STANDARD(S)

None

E. SAMPLE PREPARATION

Intact tissue samples should be stored frozen in at least a -20 ºC freezer until the DNA is extracted.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

a. Fish Genomic DNA Isolation

Note: this procedure may vary slightly, depending upon the kit manufacturer's instructions, so it is critical to be familiar with any important notes and troubleshooting procedures.

i. With a sterile razor blade, cut and weigh out 30 - 40 mg of muscle tissue into a 1.5 mL microcentrifuge tube. It is recommended to cut the tissue into small pieces to ensure more efficient cell lysis.

Note: It is critical to use different sterile scalpels for different tissues in order to prevent cross contamination.

ii. Prepare positive control by selecting an authenticated tissue for inclusion in extraction set. With a sterile razor blade, cut and weigh out 30 - 40 mg of authenticated muscle tissue into a 1.5 mL microfilterfuge tube. It is recommended to cut the tissue into small pieces to ensure more efficient cell lysis.

iii. The negative control or DNA extraction blank will also be included with every DNA extraction set, it will consist of an empty microcentrifuge tube taken through the extraction procedure. It is essentially a reagent blank and will indicate contamination in the extraction reagents.

2. Extraction Procedure

a. Add 180 µL of Buffer ATL to controls and samples, cap, and vortex.

b. Add 20 µL of proteinase K, mix thoroughly by vortexing, and incubate on shaking water bath at 56 ºC ± 1°C until the tissue is lysed. Lysis time varies and is usually complete in 1 - 3 hours. After incubation the lysate may appear viscous but should not be gelatinous as it may clog the DNeasyspin column.

c. Vortex for 15 seconds.

d. Add 200 µL of Buffer AL to the sample, cap, and mix thoroughly by vortexing a second time.

e. Add 200 µL of ethanol, cap, and mix again by vortexing. Alternatively, Buffer AL and ethanol may be premixed and added together in one step to save time. It is critical that the sample, Buffer AL, and ethanol are mixed immediately upon addition by vortexing or pipetting to yield a homogenous mixture. A white precipitate may form upon addition of Buffer AL and ethanol, but it does not interfere with the procedure.

f. Decant the mixture, including the precipitate, from Step f into a spin column which is placed into a 2 mL collection tube (provided with extraction kit). Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min and discard the flow-through and collection tube.

g. Place the spin column into a new collection tube (provided with extraction kit), add 500 µL Buffer AW1, and centrifuge for 1 min at ≥ 6000 x g (8000 rpm). Discard flow-through and collection tube.

h. Place the spin column into a new collection tube (provided with extraction kit), add 500 µL Buffer AW2, and centrifuge for 3 min at ≥ 20000 x g (14000 rpm). Discard flow-through and collection tube.

NOTE: It is critical to dry the membrane of the spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the elution step. Carefully avoid any contact between the spin column and the flow-through during the previous steps. If contact occurs, empty the collection tube and centrifuge again for 1 min at 20,000 x g (14,000 rpm). It is also an option at this point to incubate the spin columns as well as the Buffer AE at 37ºC ± 1°C for 30 minutes prior to performing elution step.

i. Place the spin column in a clean microcentrifuge tube (not provided with extraction kit) and pipet 200 µL of Buffer AE directly onto the filter membrane.

j. Incubate at room temperature for 1 min and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute the DNA.

NOTE: For maximum DNA yield, it is possible to repeat the elution step a second time either into the original microcentrifuge tube or into a new microcentrifuge tube to avoid diluting the original eluate. Alternatively, it is permissible to add a lower volume of Buffer AE to increase the subsequent DNA concentration in the eluate.

k. Discard the filter and retain the eluate, which contains the extracted DNA for use as a PCR template. Store at ≤ -20 ºC until further use.

3. Amplification of COI Gene

a. Thaw master mix and aliquot 10.5 µL into individual PCR tubes.

b. Using a micropipettor, transfer 1.0 µL of extracted DNA template (from step F.2.k. of procedure) into each PCR master mix tube, pipet up and down several times to mix, and close the lid. Also, include a PCR blank to which no template DNA is added. Spin briefly in mini-centrifuge to return contents to bottom of tube.

c. Load all sample tubes onto the thermal cycler.

d. Access the appropriate temperature program. The parameters for the program are as follows:

i. 1 cycle of denaturation at 94 ºC for 2 minutes.

ii. 35 cycles of 94 ºC for 30 seconds, 55 ºC for 40 seconds, and 72 ºC for 1 minute.

iii. 1 cycle of final extension at 72 ºC for 10 minutes.

iv. Cool to 4 ºC and remain on thermal cycler until removal for storage or further processing.

e. Run program on thermal cycler.

4. Verifying PCR Product

   Note: This step is optional, but highly recommended prior to moving forward with the cycle sequencing PCR.

   Note: The instrument parameters may be optimized to ensure system suitability.

   a. Plug the base of the electrophoresis power supply into an electrical outlet.

   b. Remove precast gel from its package and remove the plastic comb from the gel. In order to visualize the PCR product on the agarose gel, dilute sample and DNA ladder with sterile water. Transfer to the gel.

   c. Run gel at appropriate voltage (~ 250 - 300 V) to ensure sufficient movement of DNA in the gel.

   d. Verify that all standard tissues and samples contain a band at or around ~ 650 base pairs (bp). If there is no band, this indicates an unsuccessful PCR reaction. Negative controls should have no visible bands. It is recommended to troubleshoot the contributing factors and repeat from step F.2.

   e. Dispose of gel in designated and secure waste container.

5. PCR Product Cleanup

   a. Using a micropipettor, pipet 2 µL of ExoSAP-IT into a clean microcentrifuge PCR tube and then add 5 µL of PCR product.

   b. Incubate mixture on thermal cycler at 37°C for 15 minutes followed by 80°C for 15 minutes.

   c. Remove from thermal cycler and store at ≤ -20°C indefinitely.

6. Performing Cycle Sequencing Reaction

   a. Thaw master mix and aliquot 9.0 µL into individual PCR tubes.

   b. Transfer 1.0 µL of PCR product for each sample into a tube of sequence master mix and close the lid. Mix thoroughly and then spin down briefly.

   c. Load all tubes onto the thermal cycler.

   d. Turn on cycler and access the appropriate temperature program. The parameters for the program are as follows:

      i. 1 cycle of denaturation and activation of Taq polymerase at 96 ºC for 2 minutes.

      ii. 30 cycles of amplification at 96 ºC for 30 seconds, 55 ºC for 15 seconds, and 60 ºC for 4 minutes.

      iii. Cool to 4 ºC and remain on thermal cycler until removal for storage or further processing.

e. Run program on thermal cycler.

7. Sequence Reaction Cleanup

a. Remove either the appropriate number of filter cartridges or 96-well short plates from cold storage. If using the 96-well short plates for cleanup, remove the bottom and top adhesive tapes from the plate at this time.

Note: remove the bottom adhesive tape first and ensure that the plate remains horizontal to avoid losing any gel.

b. Centrifuge the filter cartridges at 850 x g for 3 minutes. Discard the microcentrifuge tubes which contain the eluate and transfer the cartridges to new microcentrifuge tubes. If using a 96-well plate, stack plate on top of 96-well waste plate and place assembly on a cushioned centrifuge carrier. Centrifuge plate at 850 x g for 2 minutes. Discard eluate.

c. Add the PCR product directly to the gel bed of the cartridges or add directly to center of each well in the 96-well plate.

d. Centrifuge the filter cartridges at 850 x g for 3 minutes or the plate for 2 minutes. Retain eluate.

e. Add 10 µL of Hi-Di™ Formamide to each samples and pipet to mix.

Note: If using filter cartridges, it is permissible to centrifuge the 96-well plate in order to remove bubbles from the samples.

f. Analyze on genetic analyzer, according to manufacturer's instructions.

8. Sample Set

Each sample set must contain one QA sample/20 samples:

a. DNA extraction blank.

b. PCR blank

c. Positive control (authenticated fish standard).

d. Intra-laboratory check sample (if needed)

e. Samples

G. CALCULATIONS / IDENTIFICATION

1. Analysis and Reporting of Sequencing Results

a. Retrieve raw sequence (ABI format) data files from genetic analyzer. One way to do this is using a flash drive.

b. Transfer file to sequence analysis workstation.

c. Using analysis software (for example, BioNumerics or equivalent), align all forward and reverse sequences into consensus sequences for all samples. In the case the negative control contains an identifiable DNA sequence, the set shall be re-analyzed.

d. In the case of all consensus sequences, verify that bidirectional reads contain at least 500 basepairs with < 2% ambiguous bases or that unidirectional (i.e. either single forward or reverse only) reads exhibit high quality characteristics according to the analysis software.

e. Establish the identity of all the samples and positive control using, in order of preference, one of the three databases:

   i. Internally-generated sequences from authenticated and vouchered specimens

   ii. Sequences from the Barcode of Life Datasystems (BOLD) collection at the University of Guelph

   iii. The nucleotide database of the National Center for Biotechnology Information (NCBI) Genbank collection

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Protective eyewear, lab coat, and gloves are recommended at all times.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
POP-7™	Neurotoxic compound	Use protective gloves when handling.
Ethanol	This solvent may be flammable and may produce toxic effects to skin, eyes, and the respiratory system.	Use reagents in an efficient fume hood away from all electrical devices and open flames. Use approved gloves and protect skin from exposure.

3. Disposal Procedures

Follow federal, state and local regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. Negative controls - no band present on the agarose gel of the PCR product check and no resulting DNA sequence from the capillary electrophoresis.
   b. Positive controls - the identity of the positive control tissue must be confirmed by the COI sequence.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Consensus sequence length	≥ 500 bp with < 2% ambiguities
b. Database match quality	≥ 98%
c. Blank	no evidence of identifiable DNA

3. Intralaboratory Check Samples
   a. System, minimum contents.
      i. Frequency: One per week per analyst when samples analyzed.
      ii. Records are to be maintained.
   b. Acceptability criteria.
      Refer to I. 1.
      If unacceptable values are obtained, then:
      i. Investigate following established procedures.
      ii. Take corrective action as warranted.

J. APPENDIX

1. References
   a. DNeasy® Blood & Tissue Handbook. July 2006. Qiagen.
   b. Handy SM, JR Deeds, NV Ivanova, PDN Hebert, RH Hanner, A Ormos, LA Weigt, MM Moore, and HF Yancy. 2011. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. Journal of AOAC International 94(1):1-10.

2. IUPAC Ambiguity Code

Source:

IUPAC Code	Meaning
M	A or C
R	A or G
W	A or T
S	C or G
Y	C or T
K	G or T
V	A or C or G
H	A or C or T
D	A or G or T
B	C or G or T
N	G or A or T or C

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff.