

**United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Fish Speciation by PCR/Sequencing of Mitochondrial Cytochrome Oxidase I (COI) Gene		
Revision: NA	Replaces: NA	Effective: 03/09/2012

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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 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.

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 distinguish between muscle tissue originating from catfishes and all other types of bony fish.

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 10 and 1000 μ L, 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.

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- 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 °C 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, 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. The stability time frame of the solution is dependent upon the expiration date of the components used or the listed expiration date, whichever is soonest.

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

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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. Ethanol (EtOH) - At least 96.0%, 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. Platinum® Taq Polymerase
- ii. 10X PCR Buffer, Minus Mg
- iii. 50 mM Magnesium Chloride
- iv. 100 bp DNA Ladder - Cat. No. 15625-019, Invitrogen.
- v. ExoSAP-IT® - Cat. No. 78200 200 uL, Affymetrix.
- vi. BigDye® Terminator v3.1 Cycle Sequencing Kit - Cat. No. 4337457, Applied Biosystems.
- vii. Performa®-DTR Gel Filtration Cartridges – Cat. No. 42453, EdgeBio.
- viii. Performa®-DTR V3 96-Well Short Plates – Cat. No. 63387, EdgeBio.
- ix. POP-7 Polymer for 3130 DNA analyzers – Cat. No. 4352759, Applied Biosystems.
- x. 5X Sequencing Buffer - Cat. No. 4336697, Applied Biosystems.
- xi. 3130xl Capillary Array - 36 cm , Cat. 4315931, Applied Biosystems.
- xii. Hi-Di™ Formamide - Cat. No. 4311320, Applied Biosystems.

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2. Solutions

a. 50 μ M COI Primer Stock Mixes :

Re-suspend each primer (from the pellet and still in its original tube) in approximately 500 μ L of sterile water. The exact volume is dependent upon original manufacturer concentration. Store primers for up to eighteen months at $\leq -20^{\circ}\text{C}$ while not in use.

b. 10 μ M COI Primer Working Solutions:

Pipette 20 μ L of primer stocks into separate 0.5 mL microcentrifuge tube and dilute to 100 μ L with sterile water. Store both solutions for up to eighteen months at $\leq -20^{\circ}\text{C}$ 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 $\leq -20^{\circ}\text{C}$ while not in use.

d. 10 μ M Sequencing Primers:

Re-suspend primers (from the pellet and still in their original tubes) in approximately 200 μ L of sterile water. The exact volume is dependent upon original manufacturer concentration. Store primers for up to eighteen months at $\leq -20^{\circ}\text{C}$ while not in use.

e. COI PCR Master Mix

i. Thaw Primers A and B, Sterile H₂O, 10% trehalose solution, Buffer, MgCl₂, 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.

iii. With a micropipettor, dispense 10.5 μ L of master mix into microcentrifuge PCR tubes until all of the solution is used. Either use master mix immediately or store in freezer at $\leq -10^{\circ}\text{C}$ for up to six months.

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Reagent Name	Per 1 Reaction (µL)	Per 100 Reactions (µL)	Final Concentration
10% trehalose	6.25	625	5%
Sterile water	2.0	200	---
10X Buffer	1.25	125	1X
50 mM MgCl ₂	0.625	62.5	2.5 mM
10 µM Primer A	0.125	12.5	100 nmol
10 µM Primer B	0.125	12.5	100 nmol
10 mM dNTP mix	0.062	6.2	50 nmol
Taq polymerase (5 U/mL)	0.06	6.0	0.6 U
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:

Reagent Name	Per 1 Reaction (µL)	Per 100 Reactions (µL)	Final Concentration
10% Trehalose	5.0	500	---
Sterile water	0.875	87.5	---
10 µM M13F OR M13R	1.0	100	0.5 µM
5X Sequencing Buffer	1.875	187.5	---
Big Dye™	0.25	25	---
Total	9.0	900	---

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D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent upon the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

- a. Vouchered Specimens of a channel catfish (*Ictalurus punctatus*), a blue catfish (*Ictalurus furcatus*), and a channel x blue hybrid are provided by the Georgia Department of Natural Resources, University of Georgia, or an equivalent source. Store all standards in at least a -20 °C freezer that does not go through freeze-thaw cycles. Keep standards in an air-tight container/wrap preferably vacuumed-sealed to prevent freezer burn. When appropriately stored, standards are stable indefinitely.

E. SAMPLE PREPARATION

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

F. ANALYTICAL PROCEDURE

1. Preparation of Controls

- 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 described therein.

- i. 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 1.5mL microfilterfuge tube. It is recommended to cut the tissue into small pieces to ensure more efficient cell lysis.
- ii. 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. 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

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troubleshooting procedures described therein.

- b. 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.

- c. Add 180 μ L of Buffer ATL to controls and samples, cap, and vortex.
- d. Add 20 μ L of proteinase K, mix thoroughly by vortexing, and incubate on shaking water bath at $56\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$ 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 DNeasy spin column.
- e. Vortex for 15 seconds.
- f. Add 200 μ L of Buffer AL to the sample, cap, and mix thoroughly by vortexing a second time.
- g. Add 200 μ L 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.
- h. Pipet 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 $\geq 6000 \times g$ (8000 rpm) for 1 min and discard the flow-through and collection tube.
- i. Place the spin column into a new collection tube (provided with extraction kit), add 500 μ L Buffer AW1, and centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm). Discard flow-through and collection tube.
- j. Place the spin column into a new collection tube (provided with extraction kit), add 500 μ L Buffer AW2, and centrifuge for 3 min at $\geq 20000 \times g$ (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 $20,000 \times g$ (14,000 rpm). It is also an option at this point to incubate the spin columns as well as the Buffer AE at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 30 minutes prior to performing elution step.

- k. Place the spin column in a clean microcentrifuge tube (not provided with

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extraction kit) and pipet 200 μ L of Buffer AE directly onto the filter membrane.

- I. Incubate at room temperature for 1 min and then centrifuge for 1 min at $\geq 6000 \times g$ (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 or into a new microcentrifuge tube to avoid diluting the original eluate.

- m. Discard the filter and retain the eluate, which contains the extracted DNA for use as a PCR template. Store at $\leq -20 \text{ }^{\circ}\text{C}$ until further use.

3. Amplification of Mitochondrial Cytochrome Oxidase I Gene

- a. Remove the desired number of tubes of PCR master mix from storage and allow them to thaw on ice.
- b. Using a micropipettor, transfer 1.0 μ L of extracted DNA template (from step F.1.I. of procedure) into each PCR master mix tube, pipet up and down several times to mix, and close the lid. Spin briefly in mini-centrifuge to return contents to bottom of tube.
- c. Add 1.0 μ L of DNA from an authenticated standard to serve as a positive control into one PCR tube and 1.0 μ L of the DNA extraction blank into a second PCR tube to serve as a DNA extraction negative control, and nothing to a third PCR tube to serve as a PCR negative control. Pipet up and down several times to mix and close the lid. Spin briefly in mini-centrifuge to return contents to the bottom of tube.
- d. Load all sample tubes onto the thermal cycler.
- e. Access the appropriate temperature program - an example of a possible file name: Catfish Cytochrome Oxidase I PCR. The parameters for the program should be as follows:
 - i. 1 cycle of denaturation at $94 \text{ }^{\circ}\text{C}$ for 2 minutes.
 - ii. 35 cycles of $94 \text{ }^{\circ}\text{C}$ for 30 seconds, $55 \text{ }^{\circ}\text{C}$ for 40 seconds, and $72 \text{ }^{\circ}\text{C}$ for 1 minute.
 - iii. 1 cycle of final extension at $72 \text{ }^{\circ}\text{C}$ for 10 minutes.
 - iv. Cool to $4 \text{ }^{\circ}\text{C}$ and remain on thermal cycler until removal for storage or further processing.
- f. 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.

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- a. Plug the base of the electrophoresis power supply into an electrical outlet.
 - b. Remove precast gel from its package, prerun according to manufacturers' recommendations, and remove the plastic comb from the gel. In order to visualize the PCR product on the agarose gel, it is necessary to load between 1-3 μL of sample and 1 μL of DNA ladder. Make up the difference to 10 μL with sterile water and mix together on a portion of parafilm prior to loading.
 - c. Run gel at appropriate voltage (~ 250 - 300 V) to ensure sufficient movement of DNA in the gel (typically 15 min).
 - d. View and photograph gel under UV light on documentation system. All standard tissues and samples should contain a band at or around ~ 650 base pairs (bp). If there is no band, this indicates an unsuccessful PCR reaction. 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 $\leq -20^\circ\text{C}$ indefinitely.
6. Performing Cycle Sequencing Reaction
- a. Remove the desired number of tubes of sequencing master mix from storage and allow them to thaw on ice. Use equal numbers of tubes of master mix that contain the forward and reverse primer, as each sample will be sequenced in both directions.
 - 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 - an example of a possible file name: Sequencing PCR. The parameters for the program should be as follows:
 - i. 1 cycle of denaturation and activation of Taq polymerase at 96 °C for 2 minutes.
 - ii. 30 cycles of amplification at 96 °C for 30 seconds, 55 °C for 15 seconds, and 60 °C for 4 minutes.
 - iii. Cool to 4 °C and remain on thermal cycler until removal for storage or further processing.

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- 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 μ L of Hi-Di™ Formamide to each samples and pipet to mix.
 - 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. Test samples to be analyzed up to 20 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), align all forward and reverse sequences into consensus sequences for all samples including the positive control. In the case that the negative control contains a detectable DNA sequence that indicates a contaminant, the entire set should be re-analyzed until this is no longer the case.

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- 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

<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
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 local, state and federal guidelines for disposal.

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.

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- 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 contaminant 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.

4. Sample Condition upon Receipt - Frozen

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.

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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 Division.