

## Chapter 5

# Introducing Students to Conservation Genetics Using Sturgeon Caviar

*Kathleen A. Nolan, Tony Catalano, Phaedra Doukakis, Vadim Birstein, and Rob DeSalle*

Kathleen A. Nolan  
St. Francis College  
180 Remsen Street  
Brooklyn, NY 11201

Anthony Catalano  
St. Francis College  
180 Remsen Street  
Brooklyn, NY 11201

Phaedra Doukakis  
Vadim Birstein  
Rob DeSalle  
American Museum of Natural History  
Central Park West at 79th Street  
New York, NY 10024

Kathleen A. Nolan is an Assistant Professor in Biology at St. Francis College, Brooklyn, NY. She is a Visiting Scientist at the American Museum of Natural History in the laboratory of Rob DeSalle. (knolan@worldnet.att.net)

Tony Catalano is a Biology student at St. Francis College who helped to develop this lab exercise. He is interested in pursuing a career in forensic science.

Phaedra Doukakis is a Biology Ph.D. student at Yale University who laid the groundwork for this lab exercise.

Vadim Birstein is the director of the Sturgeon Society, New York, NY.

Rob DeSalle is the curator of the Molecular Biology Lab in the Department of Entomology at the American Museum of Natural History in New York, NY, and a Professor at Yale University.

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## Introduction

Most museums of natural history have as one of their missions uncovering the relatedness among species. Traditionally, scientists have used measurements of physical characteristics, embryology, and the fossil record to help determine this relatedness. Many scientists are now using molecular biology tools in addition to these traditional approaches.

The Molecular Biology Laboratory in the Department of Entomology at the American Museum of Natural History in New York City is interested in uncovering relatedness among many different types of species (not just insects!). For example, molecular approaches have been used to assist in the construction of a “family tree” (phylogeny) of 25 sturgeon and paddlefish species (Order Acipenseriformes). These are the caviar-producing species.

The three species that produce the caviar that is most often found in U.S. delis (and now over the Internet) are the beluga (*Huso huso*), the sevruga (*Acipenser stellatus*) and the osetra (*Acipenser gueldenstaedti*). All sturgeon species are suffering from over-fishing and many are trying to survive despite environmental degradation. The land-locked Caspian Sea commercially-fished species (the three mentioned above) have perhaps received the most publicity regarding their exploitation.

As caviar commands a high price in the marketplace, these fish are sometimes illegally caught. All sturgeon species were placed on the Convention for Trade in Endangered Species (CITES) list in 1998. What is worse, is that sometimes caviar from the more “commonly available” sturgeon are replaced by even rarer species. The rationale behind being able to identify “unknown” or “mistakenly labeled” caviar might lead to stricter laws against illegal sales of caviar from endangered species in the future.

## PCR of Caviar

When female sturgeon are caught, they are first stripped of their roe. The fish eggs are packed carefully in salt to add to the flavor and so that they will keep for several months in an unopened jar. After the salt is added, the roe is kneaded in a certain fashion---this is the “art” to packing caviar (Cullen, 1999). When the jars are opened, the eggs should keep for several weeks if tightly covered. (I have isolated DNA from caviar that has been several months old--if tightly covered and refrigerated, the eggs appear to keep their shape and integrity, which is necessary to isolate DNA from an intact egg.)

Mitochondrial DNA genes have been sequenced for each of these species, and primers for amplifying these genes have been synthesized (Birstein and DeSalle 1998).

Experiments have been conducted in which single caviar eggs obtained from jars purchased in Manhattan delis have been tested for the three commercially available species of origin. It was ascertained through PCR analysis that a large percentage of these jars (23%) were “mislabelled” (DeSalle & Birstein 1996). In other words, the jars contained either: (a.) Species that should not have been fished for because they were endangered or (b.) Caviar that should have been sold at a lower price. As one of my Russian students put it, “There is a lot of fake caviar out there.” Species ID kits are being developed for use in conservation and wildlife forensics. It is thought that, since eggs from endangered species have been detected, illegal fishing of endangered species has occurred. The students who elect to participate in this laboratory exercise could thus contribute to a database on the occurrence of this and, in effect, be conducting forensic science.

### Materials

Sturgeon caviar. Buy inexpensive black caviar from a food import deli.

Sevruga and osetra are the least expensive, and costs about \$25 for a half ounce. Caviar is also now being sold over the Internet. This should yield hundreds of eggs, which are a little larger than the head of a pin.

Gloves

Quanta-Genomic™ Kit--QUKG-50 (this kit provides enough material for 50 samples): Quantum Biotechnologies Inc., 1801 de Maisonneuve Blvd., West 8th floor, Montreal, Quebec, Canada H#H 139, Tel: 888-DNA-KITS--514-935-2200---Fax: 888-688-3785--514-935-7541, e-mail: info@gbi.com, Internet: <http://www.quantumbiotech.com>

100% ethanol

70% ethanol

RNAse free water --Amersham-Pharmacia Biotech--1-888-573-4732, cat. #US 70783 (contains .01% diethylpyrocarbonate) \$58 for 1 liter, \$35 for 500 ml.

Distilled water

dNTP's (make a master mix of 10mM each of the 4 dNTP's), dNTP Kit - Amersham-Pharmacia Biotech, cat. #27203201, \$67.

PCR buffer (10X) (from Perkin Elmer to go with AmpliTaq. This buffer should contain 15 mM magnesium chloride. Some types of PCR buffers are not supplemented with magnesium chloride---in that case, it must be added separately.)

**Primers** are ordered going from the 5' to the 3' ends. In this exercise, we will use three primers: B72 and S2A should amplify a 150 base pair fragment of a cytochrome b gene that has been sequenced and found to be in all sturgeon. When S2 is used with S2A, only a cytochrome b gene fragment (approximately the same size) specific to sevruga sturgeon (*Acipenser stellatus*)

should be amplified. We ordered ours from Operon Technologies, 1-800-688-2248. There is what is called a \$5 set-up fee and the cost is 60 cents per base. The primers are reconstituted upon arrival with RNase free water. These come dehydrated in pico to nanomoles quantities, and should be diluted to 1-10mM. Each primer will vary slightly in concentration when it is shipped to you. The primer is further diluted to nanomole quantities when it is used in the PCR reaction mix.

**Primer 1 (B72) GCCTACGCCATTCTCCG**

**Primer 2 (S2A) CCTCCAATTCATGTGACTACT**

**Primer 3 (S2) GGAGTCCTAGCCCTCCT**

Taq polymerase (We use AmpliTaq from PE Biosystems, 1-800-327-3002, Cat. # N808-0160, \$155. This comes with the required PCR buffer (10X) supplemented with 15 mM magnesium chloride. (Some types of PCR buffers are not supplemented with magnesium chloride, in that case, it must be added separately.)

Agarose

2 X TBE buffer pH 8.0 (Tris-boric acid-EDTA buffer--can be purchased from Carolina Biological, 1-800-334-5555, cat #219027, \$25 for 500 ml, or made up yourself - see recipe below - you would first make up a 10X solution, and then dilute to 1X as needed.)

To make one liter of 10X TBE buffer:pH 8.0 add the following to 700 ml of distilled water in a 2-liter flask:

1 g of NaOH

108 g. of Tris base

55 g boric acid

7.4 g of EDTA

Stir to dissolve; bring to volume. (Micklos and Freyer, 1990)

NaOH (to adjust pH to 8.0 if necessary)

Hydrochloric acid (to adjust the pH)

To make 100 ml of 6X loading dye, dissolve:

0.25 g bromophenol blue

0.25 g xylene cyanol

in 49 ml of water. Stir in 50 ml of glycerol. (Micklos and Freyer, 1990)

0.025% methylene blue or

ethidium bromide (5 mg/ml) - HANDLE WITH CAUTION!! - (See Micklos and Fryer (1990) to learn how to handle this mutagen.)

mineral oil

ice bath

minicentrifuge

micropipettors (1-20  $\mu$ l and 1000  $\mu$ l)

micropipettor tips (1-20  $\mu$ l and 1000  $\mu$ l)

(Some labs have micropipettors that range from 0-10  $\mu$ l; if this is the case, than an additional tip size would be needed.)

## PCR of Caviar

Eppendorf tubes (0.5 ml and 1.5 ml)

Polymerase Chain Reaction (PCR) machine,  
or

Water baths set at the appropriate temperatures for manual PCR (this is untested).

Gel electrophoresis set ups (trays, combs, gel chambers, power supplies)

Trays for staining with methylene blue and/or

Ethidium bromide

Plastic Rubber-maid type containers with covers in which to place gels for staining.

Photography equipment (optional)

(In this laboratory both Polaroid and a Kodak digital photography set-up in use with a MacIntosh computer are used for photography). Life Technologies, 1-800-828-6686, distributes the Kodak Digital Science EDAS 120. A Polaroid specifically for use with UV can be purchased from Carolina Biological, cat. #213699, \$450.

Light box--UV for viewing ethidium bromide stained gels and/or

White light for viewing methylene blue stained gels

### Notes to Instructor

**Level of difficulty:** Upper undergraduate genetics course

**Time required to prepare and set up:** five to eight hours

**Time required for students to perform exercise:**

Two hours to isolate DNA and to make dilutions for the control primers; three hours for the PCR reaction to run; one hour to run the gel.

A repeat of the above times is needed for the reactions with the species-specific primers.

I suggest that you take two weeks to complete this exercise; one week to do the control, and the second week to do the species-specific reactions.

You will need to make a list of the total number of spots that are available on your PCR machine(s). Each student (if working individually) will need three spots; however, I suggest that one dilution (1:10) will probably work. Once you have the number of spots matched to the number of students you have, make a similar list for the number of reactions, number of students, and wells on a gel available based on type of comb and number of gel boxes.

The recipe for the reaction mix as given in the protocol is for one student. You need to multiply these amounts by the number of students you have, plus extra for negative controls. The number of negative controls will be determined by the number of wells you have on your gel, and how many students are using each gel.

This experiment has been successfully tested with the species-specific *Acipenser stellatus* primers. There are primers available for the *Huso huso* caviar, but this caviar is twice as expensive as the sevruga, and therefore probably not practical for this exercise. Also, the osetra primers will hybridize to species closely related to the *Acipenser gueldenstaedti* so they are considered to be species-specific enough for this exercise. The most precise way to determine whether a certain caviar egg is from a specific species is to sequence the DNA, which goes beyond the scope of this exercise.

## Student Outline

### Protocol--Week One

#### Isolation of DNA from single caviar eggs

##### Lysis and homogenization step

**Note: The Quantum kit components come in four bottles labeled GEN I through GEN IV. (These kits contain proprietary solvents, which the company representatives, when called on the phone, would not reveal what they were. A good biological techniques book (Robyt and White, 1987, is one example) should describe how traditional ingredients such as detergents and ethanol work in lysing cells and precipitating DNA.)**

1. Add 300  $\mu$ l of GEN I to each of two 1.5 ml microfuge tubes.
2. Add one sturgeon egg from a microfuge tube marked "S" for sevruga to the first tube with a yellow 20  $\mu$ l pipette tip, and "smash the eggs open" against the side of the tube. You should see white material oozing from the egg. In the second tube repeat the process with an egg from a microfuge tube marked "O" for osetra.
3. Add 300  $\mu$ l of GEN II to each tube and mix by inverting the tube several times.
4. Incubate the tubes at 55° C for 30 minutes.
5. Add 150  $\mu$ l of GEN III to each tube and mix vigorously by inverting the tubes twenty times.
6. Leave the tubes at room temperature for 10 minutes, mixing them occasionally by inverting them.
7. Centrifuge the tubes at high speed in a microfuge (14,000 RPM) for 5 minutes.
8. Transfer the supernatants to fresh labeled tubes, and discard the tubes with the pellets.
9. Add 450  $\mu$ l of isopropanol to each tube of supernatant and mix well.
10. Centrifuge the tubes at high speed (14,000 RPM) for 5 minutes. Discard the supernatants.
11. Rinse the precipitate in each tube with 200  $\mu$ l of 70% ethanol. Centrifuge the tubes for 5 minutes. Discard the supernatants.
12. Allow the pellets to air dry until there are no drops of alcohol left--this step may be aided with a hair dryer at a low setting.

## PCR of Caviar

13. Resuspend each pellet in 20  $\mu$ l of RNase-free water.

### PCR Amplification

1. You may warm the sample for a few minutes in a 55°C water bath, if the DNA appears to not have gone into solution.
2. Since you are not sure of the quantity of DNA that you have isolated, and the PCR reaction is DNA concentration-dependent, it will be necessary for you to do a dilution series of your DNA. (You need to see how many PCR machines you have, or how many spots you have available to you on a PCR machine to see if it is practical for your class to do the amplifications of every dilution. If you don't have a lot of room on the machines, use a 1:10 dilution of the DNA for the PCR reaction.)
3. Set up two 1.5 ml tubes for the sevruga DNA and label them 1:10, 1:100. Repeat the process for the osetra DNA.
4. Place one  $\mu$ l of the appropriate DNA in the appropriately labeled tube. Add the RNase-free water to make the dilution. (Check with your instructor first to make sure that you are adding the correct amount of water.)

5. To set up the PCR mix:

Add 1  $\mu$ l of the appropriate dilution to a 0.5 ml (PCR) tube. You should have three PCR tubes: one for the undiluted DNA, and the other two for the two dilutions that you made.

Next, add 24  $\mu$ l of the PCR reaction mix that has been prepared by the instructor and is sitting on ice.

The control reaction mix contains the following ratios of reactants:

- 19  $\mu$ l RNase free water
- 2.5  $\mu$ l of PCR buffer supplemented with 15 mM magnesium chloride
- 2.5  $\mu$ l of DNTP's (10 mM each)
- 0.1  $\mu$ l of primer 1 (B72)
- 0.1  $\mu$ l of primer 2 (S2A)
- 0.1  $\mu$ l of Taq polymerase

6. Spin tubes briefly at top speed in the microfuge for a few seconds.
7. Add two drops of mineral oil from a dropper bottle to each tube.
8. Spin tubes briefly at top speed in the microfuge for a few seconds.

- Place the PCR tubes in the PCR machine. Run the PCR reaction under the following parameters:

35 cycles of:

94°	1 minute
48°	1 minute
72°	1 minute

- When the samples are finished, place them at 4° C until you have time to complete the electrophoresis.

### **Electrophoresis.**

- Place 1 gram of agarose into a 125 ml Erlenmeyer flask. Add 50 ml of 1X TBE buffer.
- Microwave the solution until it boils (about a minute and a half). Alternatively, heat the agarose in buffer to boiling on a hot plate. Make sure that all the agarose is dissolved, but don't burn it. (What percentage gel would this be?)
- Let the gel cool at room temperature till hot but not burning to the touch. (Touch the flask to your cheek!)
- At this point, add 0.5 µl of a 5 mg/ml solution of ethidium bromide to the agarose. CAUTION!! Since ethidium bromide intercalates between the bases of DNA, it is a potential carcinogen. Handle with care. Omit this step if you are using the methylene blue method of DNA staining.
- Cast the gel according to the directions that come with your gel tray--you can use tape to seal the ends, or some trays come with rubber "gaskets" that fit over the ends and effectively seal it. Place your tray on a level surface. Place a 8-12 well comb into the tray. Pour the gel quickly. Push any bubbles to the side with a yellow micropipet tip.
- The gel should be hardened after about 20 minutes--it will turn opaque and you may see wavy lines running through it.
- Pull out the comb by wiggling it gently as you pull--this takes a little practice. Take off the tape or the rubber gaskets.
- Place the gel in an electrophoresis chamber. Cover the gel with 1X TBE. You should just barely cover the gel-- the less buffer, the less resistance to the current. As you increase the amount of buffer, you will notice that by switching to the "current" or "ampere" setting, the number of amperes increases, which is undesirable because this could create too much heat and cause your gel to melt.

## PCR of Caviar

9. If necessary, you may practice pre-loading the gel with loading dye, and then run the dye a few centimeters through the gel by turning on the voltage to 125 V.
10. Prepare to load the gel. Take a piece of parafilm around 10 cm square and place it down flat on the lab bench.
11. Add 2  $\mu$ l of dye as dots spaced apart on the parafilm--one for each sample that you will load.
12. Spin tubes briefly at top speed in the microfuge for a few seconds and take off as much mineral oil as possible with a micropipette. You must do this very slowly and carefully in order to avoid taking out your sample along with the oil.
13. Make a key of the order of your samples that you will be loading into your gel in your notebook.
14. Mix your samples with loading dye, which: (a) contains glycerol which weighs the samples so that they do not float out of the gel and (b) contains two dyes that will separate upon migration through the gel. Add 8-10  $\mu$ l of each sample to the blue loading dye dot and mix by pipetting up and down once. Take the sample from the bottom of the tube so that you won't get any mineral oil in your sample. (The mineral oil won't mix with the dye.)
15. Carefully add your samples to the wells. Turn on the power supply and set the voltage at 125 volts. You may go up to 150 volts, but heating the gel for too long a period of time may distort it.
16. You will notice that the loading dye will separate out into a dark blue and a light blue band. The faster-moving dark blue band will co-migrate with a DNA fragment that is approximately 300 base pairs in length, and the slower-moving light blue band will co-migrate with a DNA fragment that is approximately 9,000 base-pairs in length.
17. Turn off the power supply, remove the gel tray, and carefully slide the gel into a staining chamber.
18. If you used ethidium bromide, cover your gel with distilled water. If you want to use methylene blue for staining, cover the gel with 0.025 % methylene blue.
19. The ethidium bromide gel may be ready for photography. View the gel on the UV light box. Be sure to use a protective face shield or box cover, and determine if bright clear bands are present. If the bands are not differentiated enough from the background, destain the gel by leaving it in the distilled water for an hour to several hours.

20. If you used methylene blue, leave the gel in this solution for 30 minutes. Destain the gel by pouring off the methylene blue solution into a storage container, and running the gel under tap water for a few minutes. Leave the gel in tap water for several hours; the bands will become more visible with destaining.

## Photography

### A. Digital photography

The laboratory at the American Museum of Natural History is equipped with a Kodak camera that takes digital pictures and captures them to a computer. A more traditional set-up is the Polaroid camera. We think that every lab should aim toward a digital set-up, and try to obtain grant money for this.

If you have a digital camera, follow the directions for taking a picture. If you save the image as a TIF or PIC file, it will use up less room on your disc than the Kodak graphics save. Then you may print a copy of your photo, using the Adobe graphics program.

### B. Polaroid photography

For UV photography of ethidium bromide stained gels, use Polaroid high-speed film Type 667 (ASA 3000). Set the camera aperture to f/8 and shutter speed to B. Depress shutter for a 2-3 second time exposure.

For white light photography of methylene blue stained gels, use Polaroid Type 667 film, with an aperture of f/8 and a shutter speed of 1/125 second. (You may have to play with these settings!)

## Results

You should see nothing in your negative control lane and a nice, 150 base-pair band in your sample lanes. If you get this result, then you have successfully amplified and visualized a 150 bp fragment of a sturgeon cytochrome b mitochondrial gene!

This is your control; you will now repeat the species-specific experiment substituting the S2 primer for the B7-2 primer.

## Week Two. Species-specific DNA PCR amplification

Take your DNAs from both the osetra and the sevruga caviar and test it with the sevruga species specific primers (S2 and S2A). For these PCR amplifications use the dilution that gave you the brightest band for the control primers. This set of primers should give a positive amplification for any egg that is from the species *Acipenser stellatus*, or the sevruga caviar. Repeat from Step 5 under "PCR Amplification, substituting one tube for the three tubes with the three different dilutions. Follow the protocol through the "Electrophoresis" and "Photography" sections. Note that the **primers** and the **PCR reaction conditions** are different. Note your results.

## PCR of Caviar

The experimental or species-specific reaction mix has been prepared to contain the following ratios of reactants:

- 19  $\mu$ l RNase free water
- 2.5  $\mu$ l of PCR buffer supplemented with 15 mM magnesium chloride
- 2.5  $\mu$ l of DNTP's (10 mM each)
- 0.1  $\mu$ l of primer 1 (S2) Note that this primer is different from that in the control.
- 0.1  $\mu$ l of primer 2 (S2A)
- 0.1  $\mu$ l of Taq polymerase

The **PCR reaction conditions** are slightly different for the sevruga species-specific reactions:

- 35 cycles of:
  - 94° 1 minute
  - 55° 1 minute
  - 72° 1 minute

If the species is not *Acipenser stellatus* (or sevruga), there should be no bands that will light up. Make sure that you set up your gel so that you have one lane that contains some positive control DNA (from Week One).

Primers that are sturgeon species-specific are still being sought in order to differentiate endangered from non-endangered sturgeon species. You have just taken a step closer to becoming a wildlife forensic scientist!

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