Making Conservation Genetics Come Alive for Students

Claire Leonard[1] & Kathleen A. Nolan[2]

[1]Department of Biology
William Paterson University
300 Pompton Rd
Wayne, NJ 07470-0913
(973)595-2247; Fax: (973)595-2338
leonardc@wpunj.edu

[2]Department of Biology
St. Francis College
180 Remsen St.
Brooklyn, NY 11201
(718)489-5439; Fax: (718)533-1274
knolan@worldnet.att.net

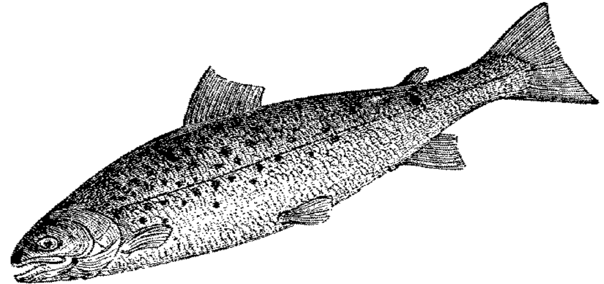
Abstract: This article describes a successful adaptation of a laboratory exercise that was presented at an Association of Biology Laboratory Education (ABLE) workshop. The workshop entailed isolating DNA from sturgeon caviar eggs, amplifying DNA using the polymerase chain reaction, and examining the DNA fragments obtained on a gel. Students from William Paterson University modified the experiment by bringing in their own egg samples of species other than sturgeon. Three different classes of students repeated the experiment, and obtained the same results each time. This experiment is fast, simple, inexpensive, fun, reliable, and repeatable. It will also teach your students about conservation genetics in an involved, exciting way.

Keywords: PCR, conservation genetics, investigative laboratory, sturgeon phylogenetics

Introduction

This article depicts a successfully performed adaptation of a lab exercise that was originally presented at the 21st Annual Association of Biology Laboratory Education Conference at the University of Nebraska-Lincoln in June, 1999 (Nolan, 2000). The title of the exercise is: *Introducing Students to Conservation Genetics Using Sturgeon Caviar.* This exercise involved using universal sturgeon and species-specific sturgeon primers to amplify DNA isolated from caviar of known and unknown origin.

Cullen (1999) describes the decline of several species of caviar producing sturgeon for commercial purposes in the Caspian Sea. American caviar producing species are also in decline in areas such as the Hudson River. There is currently a moratorium on sturgeon fishing in the Hudson River for the next forty years (Waldman, personal communication). All twenty-five species of sturgeons and paddlefishes (order Acipensiformes) are threatened by overfishing and habitat degradation (Birstein et al., 1998). Most species of sturgeon do not reproduce until they are approximately fifteen years old, which means that sturgeons are very slow-growing fish. Unfortunately, they are often harvested before they are even at this reproductive age, which contributes even more to their decline in abundance. Caviar commands a high price,



and, has become increasingly sought after. DeSalle andBirstein (1996) reported that of twenty-three lots of caviar purchased in Manhattan delis and two lots from Russia, five were incorrectly labeled, as identified by species-specific primers that have been developed. Sometimes a species of sturgeon that produces "cheaper" caviar is substituted for a species that produces more expensive caviar. Quick methods of

species identification of sturgeon as well as other organisms may reveal other misidentifications as well. Ultimately this work may put pressure on sellers and consumers alike to conserve our natural resources.

In this adaptation of the fore-mentioned lab exercise, performed at William Paterson University, other types of fish eggs were used as well as the sturgeon eggs. This is an example of an investigative laboratory exercise in that the students were able to participate in the planning of this experiment from the onset. For example, the instructor purchased the beluga caviar initially, and invited the students to "scour their refrigerators and local supermarkets for whatever type of fish eggs they could find!" One student working on a zebrafish research project brought in freshly laid zebrafish eggs.

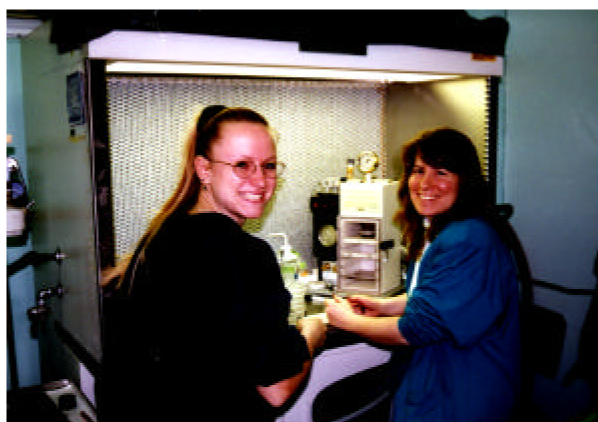


***Figure 1**. William Paterson students at work.*

The exercise was performed three times by undergraduate students from William Paterson University attending the summer 1999 Advanced PCR Technology course offered by the Biology Department and taught by Dr. Claire Leonard. This was an efficient and expedient experiment that demonstrated the technical aspects as well as the application of PCR. In addition, the most exciting aspect of this exercise is the students' enthusiasm for sleuthing. For example, one of the students titled her lab report: "*Using PCR to uncover caviar fraud*".

The techniques and methods used in the ABLE workshop were extended by the students by additional DNA extraction procedures and test materials.

Materials and Methods

- Caviar. Caviar technically means "sturgeon eggs", the color of which is always black. Paddlefish, now raised commercially, yield eggs that are brown. Many varieties of fish, such as red salmon, eggs may say "caviar" on the jar. Whitefish eggs are also black. Sturgeon caviar comes in three commercial varieties. Sevruga and osetra are the least expensive, and cost about $25 for a half ounce each. Beluga caviar is twice as expensive. You may buy caviar from delis or specialty import food stores. Caviar is also now being sold over the Internet. One half ounce should yield hundreds of eggs which are a little larger than the head of a pin. Salmon, white fish, and beluga sturgeon eggs were purchased for use for this experiment by the instructor, or were provided by the students. In our experiments, one student provided zebrafish eggs.
- Gloves
- Quanta-GenomicTM Kit. The QUKG-50 kit provides enough material for 50 DNA extractions [Quantum Biotechnologies Inc., 1801 deMaisonneuve Blvd., West 8th, Montreal, Quebec, Canada H#H 139; Tel: 888-DNA-KITS or 514-935-2200; Fax: 888-688-3785 or 514-935-7541; Internet: http///www.quantumbiotech.com; e-mail: info@gbi.com]
- Qiagen Dneasy Tissue KitTM--69504 (this kit provides enough material for 50 DNA extractions) [Qiagen Co., 1-800-426-8157]. (If you decide to use the Qiagen Dneasy Tissue KitTM, a vortex, a 55°C water bath, and a 70°C water bath will be necessary, in addition to the other materials listed.)

Subsequent materials listed are those required for use with the Quantum kit.

- 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].
- **Primers**

  Primer 1 (B72) GCCTACGCCATTCT CCG

  Primer 2 (S2A) CCTCCAATTCATGT GAGTACT

  Primer 3 (S2) GGAGTCCTAGCCC TCCTG

  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.

The American Museum of Natural History orders primers from Operon; whereas, William Paterson University orders primers from Biosynthesis, Inc. [1-800-227-0627---$1.19/base].

Usually the primers come lyophylized in nanomole concentrations. These need to be reconstituted in RNase-free water to a 25 ìmole concentration. For example, if the total amount on your primer vial is listed as **70** nanomoles, then use the following formulation:

25 ìM = 25 ìM per liter = 25 nM per ml.

$$\frac{70 \text{ nmoles}}{X \text{ ml}} = \frac{25 \text{ nmoles}}{\text{ml}}$$

$$x = 2.8 \text{ ml}$$

Reconstitute primer vial with 2.8 ml of RNase-free water.

For one PCR reaction, the primer will be diluted from 1:50 to 1:250, depending on which protocol you use.

- Taq DNA 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
- 20X TBE buffer pH 8.0 (Tris-boric acid-EDTA buffer) can be purchased from Carolina Biological [1-800-334-555; cat. # 219027; $25 for 500 ml] Or you can prepare the buffer using the following recipe. First you should prepare the 10X solution, 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 and Hydrochloric acid (to adjust pH of TBE buffer to pH 8.0 if necessary)
- 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 Freyer (1990) to learn how to handle this mutagen)
- Mineral oil
- Ice bath
- Minicentrifuge
- Micropipettors and micropipettor tips (1-20 ul and 1000 ul). (Some labs have micropettors that range from 0-10 ul; if this is the case, then an additional tip size would be needed)
- Eppendorf® tubes (0.5 ul and 1.5 ul)
- 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 are good
- Photography equipment (optional). In this laboratory both Polaroid® and a Kodak® digital photography set-up is used with a MacIntosh® computer for photography. Life Technologies (1-800-828-6686) distributes the Kodak Digital Science EDAS 120 unit. A Polaroid® unit specifically for use with UV can be purchased from Carolina Biological (cat. # 213699; $450).
- Light box. UV for viewing ethidium bromide stained gels and white light for viewing methylene blue stained gels

Notes to Instructor

Time Required for Students to Perform Exercise.

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

We suggest that you take two weeks to complete this exercise; one week to do the DNA extractions and set up the PCR, and the second week to run and stain the gels.

You will need to make a list of the total number of spots that are available on your PCR machine(s) when you have determined the number of samples you will run. A 1:10 dilution of the extracted DNA was used for this experiment. Once you have the number of spots matched to the number of students, make a similar list for the number of reactions 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. Make extra reaction mix for positive and 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.

Initially, this experiment was first tested with the universal sturgeon primers B7-2 and S2A that will amplify fragments of varying sizes in any of the 25 species of Acipensiformes (the order of sturgeons and paddlefishes). The students from William Paterson University also amplified fragment from other species unrelated to sturgeon using these universal sturgeon primers. This might not be unexpected, as sequences for these other fish species were not found in GeneBank®.

The primers S 2 and S2 A were originally thought to be species-specific *for Acipenser stellatus* (sevruga caviar) as presented in the ABLE workshop. These primers amplify a 150 bp fragment of the cytochrome b gene. However, the students from William Paterson University found that these S2 and S2A primers also amplified a 500 bp fragments from zebrafish. This was an unexpected and interesting observation.

Protocol--Week One

Isolation of DNA from Single Caviar Egg

1. **Lysis and Homogenization**. Note: The Quantum kit components come in four bottles labeled GEN I through GEN IV. These kits contain proprietary solvents and the company representatives, when contacted by phone, would not reveal their identity. A good biochemical techniques book such as Robyt and White, 1987, should describe how traditional ingredients such as detergents and ethanol work in lysing cells and precipitating DNA.
2. **DNA Extraction**. Follow the directions according to the kit using three to five sturgeon eggs for a good yield of DNA. Use a 20 ìl pipette tip to "smash open" the egg against the side of the tube with the first reagent (GEN I). You should see white material oozing from the egg. Continue to follow the protocol according to the kit directions.

PCR Amplification

Sometimes it is necessary to do a dilution series of your DNA in order to yield the optimum amount of DNA that can be amplified, but, in most cases, a 1:10 dilution has worked for us.

To set up the PCR mix you will do the following:

1. Add 1 ìl of the appropriate dilution to a 0.5 ml (PCR) tube. .
2. Next, add 24 ìl of the PCR reaction mix with the reagents in the following proportions for each sample:
   19 ml RNAse free water
   2.5 ml of PCR buffer supplemented with 15 mM magnesium chloride
   2.5 ml of DNTP's (10 mM each)
   0.1 ml of primer 1 (B72)
   0.1 ml of primer 2 (S2A)
   0.1 ml of Taq polymerase
3. Run the PCR under the following parameters for the B7-2 and S2A primers:
   1 min @ 94° C
   1 min @ 48° C
   1 min @ 72° C; repeat for 35 cycles
4. The PCR reaction conditions are slightly different for the sevruga species-specific reactions:
   (Primers S2 and S2A)
   1 min @ 94° C
   1 min @ 55° C
   1 min @ 72° C; repeat for 35 cycles

Electrophoresis.

1. Make a 2% agarose gel for the electrophoresis with 1X TBE buffer.
2. Mix your samples with loading dye, which: a. contains glycerol which weights 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. 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.
3. Run the gel at no more than 125 volts. When it is finished running, add 0.5 ìl of a 5 mg/ml solution of ethidium bromide to a staining tray of distilled water and stain for ten minutes. 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 staining.
4. If you want to use methylene blue for staining, the gel should be covered with 0.025 % methylene blue for an hour and destained overnight. If possible, there should be a few changes of water.

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. 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 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 DNA gene! This fragment may vary in size, according to species used. The William Paterson students also amplified a 150 base-pair fragment of DNA isolated from zebrafish and Romanoff whitefish eggs (Figure 2.). The students did not obtain amplification of a 150 base pair fragment with a sample of salmon eggs or a second sample of whitefish, but this was attributed to poor egg quality. The sevruga species-specific primers should yield a 150 bp band with the S2 and S2A primers only. The William Paterson students did not obtain amplification using the sevruga-specific primers with any sample except with one exception -- the zebrafish eggs. The large smudges at the bottom of the lanes are primer-dimers, which occur when unutilized primers hybridize with each other.

Discussion

The students were able to amplify a 150 base-pair fragment of DNA that was isolated from most of their samples. The same results were obtained when repeated by three different classes of students. The B7/S2A primers amplified a 150 base-pair sequence of DNA from species other than sturgeon, which indicates that this fragment could be found in more than one order of fish. The 500 base-pair fragment amplified by primers which were thought to be species-specific for *Acipenser stellatus* was an unexpected result. Further research is needed to explore consensus among this fragment and others and further sequencing work needs to be executed on all fish species.

If the students would like to use the Web to learn how to look up and analyze sequence homology, Rick Hershberger of Carlow College has developed a very user-friendly interactive web site for this purpose (www.bioactivesite.com/biocomputing/darwin2000/).

In conclusion, this experiment is:

- **student-oriented:** the students are able to have input from the beginning by bring in their own egg samples
- **investigative:** the student gets to help in the design of the experiment
- **practical and inexpensive**: it can be conducted in a finite time frame
- **reliable and repeatable**: students from three classes obtained results, and the results were practically identical among each group
- **exciting and fun**: the experiment does lend itself to a party atmosphere (especially if you serve caviar!) but on a more serious note, the students get to see "conservation genetics" in action. It feels real to them Primers that are sturgeon species-specific are still being sought in order to differentiate illegally from legally caught sturgeon. Upon completion of this experiment, the students can be told they have just taken a step closer to becoming a wildlife forensic scientist!

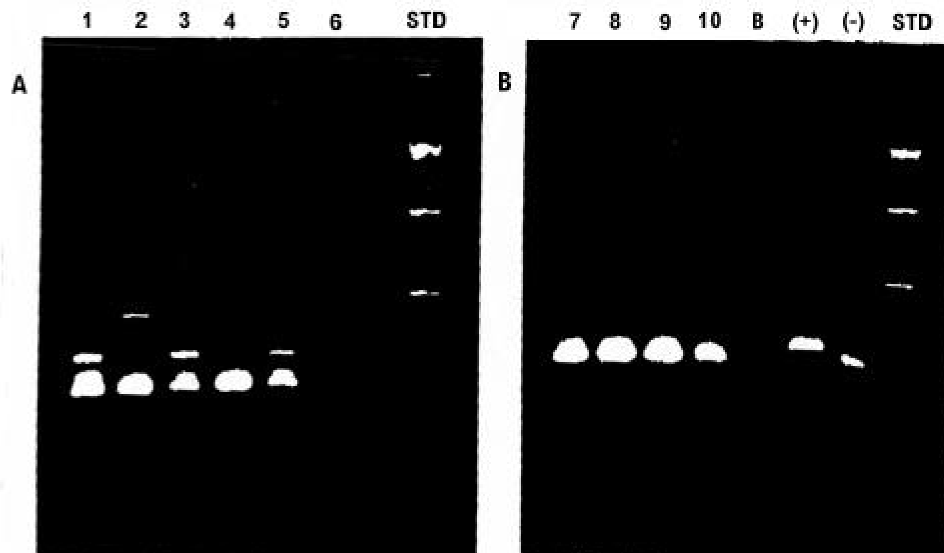


Figure 2. *Lane 1 - Zebra [B7/S2A (A)]; Lane 2 - Zebra [S2/S2A (B)]; Lane 3 - Beluga [B7/S2A]; Lane 4 - Beluga [S2/S2A]; Lane 5 - Whitefish (Romanoff) [B7/S2A]; Lane 6 - Whitefish (Romanoff) [S2/S2A]; Lane 7 - Salmon [B7/S2A]; Lane 8 - Salmon [S2/S2A]; Lane 9 - Whitefish - [B7/S2A]; Lane 10 - Whitefish [S2/S2A]; Lane B - Blank; Lane (+) - positive control; Lane (-) - negative control; Lanes STD - standards.*

References

Birstein, V., Doukakis, P., Sorkin, B., and R. DeSalle. 1998. Population aggregation analysis of three caviar-producing species of sturgeons and implications for the species identification of black caviar. Conservation Biology 12:766-776.

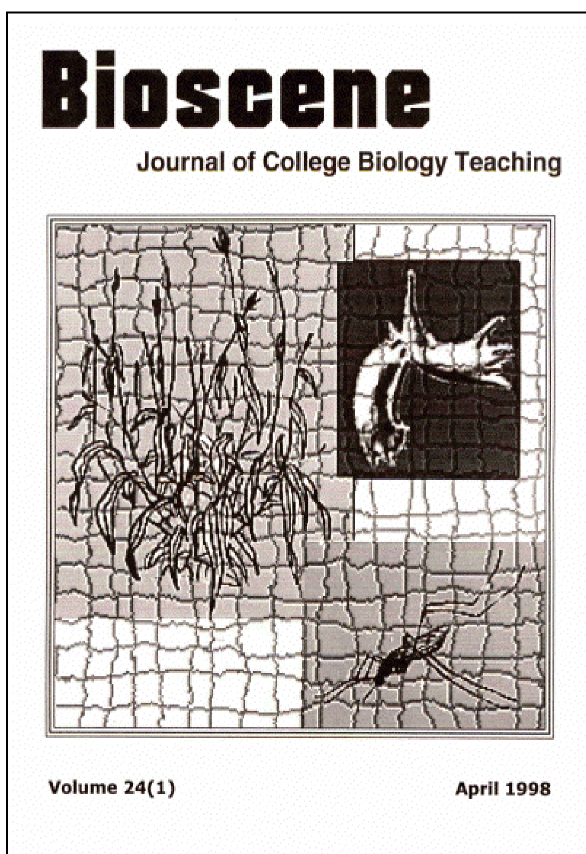
Cullen, Robert. 1999. The rise and fall of the Caspian Sea. National Geographic 195:2-35.

DeSalle, R. and V. Birstein. 1996. PCR identification of black caviar. Nature 381: 197-8

Micklos, D. A. and G. A. Freyer. 1990. DNA Science A First Course in Recombinant DNA Technology. Carolina Biological Supply Co. [ISBN 0-89278-411-3]

Nolan, K., Catalano, A., Doukakis, P. and R. DeSalle. 2000. Introducing students to conservation genetics using sturgeon caviar. *In*: Tested Studies in Biology Laboratory Education. Association of Biology Laboratory Education (ABLE) *in press.*

Robyt, J. and B. White. 1987. Biochemical Techniques: Theory and Practice. Waveland Press, Prospect Heights, Illinois. [ISBN 0-88133-556-8]



Bioscene Contributors

Do you have a manuscript, announcement, book review, labs/field studies that work, course development materials, technological advice, software reviews, curricular innovation, letter to the editor, or undergraduate research opportunity to share?

Publish your work in **Bioscene**. We prefer receiving word processing files [RTF (Rich Text Format) encouraged but Microsoft Word, Word Perfect, etc. files are acceptable] to facilitate distribution of your manuscript to reviewers and to make revisions as necessary. If you wish to attach a file to email (mulkey@biology.indstate.edu or stanleye@beloit.edu), please address the subject line as **Bioscene**. Please include graphics in an electronic format (TIFF, BMP, JPG, GIFS, or PICT formats are acceptable). Potential color cover are is always appreciated.

Your submission should include the title, author(s), name of your institution with the address, a brief abstract (200 words or less), keywords, and references cited in the manuscript in appropriate.

Please note that the deadline for the August 2000 issue is July 15, 2000.