Using the Novel Dipstick DNA Extraction Technique in a Biological Barcoding Lab

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This laboratory exercise introduces students to DNA extraction, biological barcoding, and sequence analysis; and reinforces the concept of the polymerase chain reaction (PCR). It takes advantage of the dipstick extraction technique that is cheap and fast and allows for DNA extraction and PCR setup on the same day in an 1:50 hr Introductory Biology lab. Students determine whether local businesses actually use the fish that they advertise, or if cheaper fish are being passed off as the more expensive species in order to boost profits. Different species of fish are collected from local vendors; DNA from their tissues is extracted; and a region from the Cytochrome C Oxidase I gene is amplified. After PCR, results are sent off for sequencing. The following week, chromatogram results are uploaded to NCBI, they are compared to existing genomic datasets, and conclusions are discussed in class.

Keywords: DNA Barcoding, Dipstick DNA Extraction, PCR, molecular techniques, taxonomy

Introduction

Some molecular techniques, such as PCR and gel electrophoresis, are so essential to modern biological research that they are introduced and integrated into Introductory Biology courses and laboratory exercises. Extraction of DNA material and DNA barcoding are also important techniques with wide-ranging practical applications but are not often included in such classes due to lengthy procedures. However, with the advent of more expeditious extraction methods, these procedures are well-suited for use in introductory courses. The authors use these techniques in a two-part series. The first set of lab exercises focuses on introducing students to PCR and gel electrophoresis, and the second set focuses on DNA extraction and DNA barcoding. The latter is presented here.

Although DNA barcoding has been used previously in undergraduate courses (Met et al., 2013; Butler et al., 2014; Robinson and Spindler, 2015), it has been focused toward taxonomy, biodiversity, and ecology, and those exercises utilized more expensive and lengthy DNA extraction techniques which may leave some institutions with time constraints or budgets that make their use prohibitive. The goal of this project was to introduce these techniques to a majors Introductory Biology course

with a completely new, inexpensive, and easy to perform DNA extraction methodology, dubbed the dipstick technique (Zou et al., 2017). Specifically, students study PCR, learn about DNA extraction, discover an exciting and practical field of research, and practice their analytical skills using DNA sequences.

In these exercises, students perform DNA barcoding analyses on fish samples taken from various markets, restaurants, or other vendors in town. Their objective is to determine if the DNA they extract from the tissue samples belongs to the species from which the businesses are advertising, or if it belongs to a species that is cheaper to market and has been fraudulently passed off as the original. During the first week, students will extract DNA and amplify the barcoding region (Cytochrome C Oxidase I) using PCR. The PCR product will then be sent to a local sequencing facility for analysis. During the second week, they will learn to read their resulting chromatograms and will perform BLAST searches to identify their species.

Though these exercises were initially designed for use in a college-level Introductory Biology class for majors, they can be modified to work in nonmajors labs as well as labs at the high school level, or even with summer science camps or related programs.

Student Outline

Objectives
- Describe multiple applications of DNA barcoding
- Describe the methodology involved in DNA barcoding
- Extract DNA from tissue samples
- Perform PCR to amplify a specific region of DNA
- Analyze DNA sequence data
- Use online DNA barcoding database to identify an unknown species

Prereading: Review the structure and function of DNA before coming to lab. Also, review the concepts of 1) DNA replication, 2) transcription, 3) translation, 4) electron transport chain (ETC), and 5) the process of PCR. A thorough understanding of the methodology of this lab will rely on mastery of those concepts.

For a review of the PCR procedure, please study this video:
https://www.dnalc.org/resources/animations/pcr.html

Introduction

DNA barcoding is a molecular technique used for identifying the species of an unknown individual based on its DNA. The barcoding process utilizes short segments of DNA that are present in all major groups of species. The genetic regions that are used for those groups are selected because they evolve rapidly enough that each species has accumulated enough mutation to have its own genetically distinct sequence, but not so rapidly that different individuals of the same species have too many differences to be grouped together. The genetic region used to barcode most animal species comes from a 648 base-pair segment of the cytochrome C oxidase (COI) gene. This gene is located in mitochondria, and it codes for the "I" (pronounced "one") subunit of the cytochrome C protein. You are already familiar with COI, as it is the quaternary-structure protein complex at the end of the electron transport chain (ETC) of cellular respiration. That protein complex, called either cytochrome c oxidase, COI, or complex IV, is responsible for building the proton concentration gradient across the inner membrane, as well as transferring electrons from the ETC to O_2 to form water. This region has been shown to be highly effective at identifying many different invertebrate and vertebrate animal groups, including fish. In fact, you will be using it to identify fish in this lab.

The workflow for a standard DNA barcoding identification involves 4 steps (Fig. 1). Tissue samples from the specimen must be obtained and DNA extracted from the tissue. Targeted gene regions (COI if you are identifying an animal) must then be amplified using PCR, and then those gene regions must be sequenced. Lastly, the DNA sequences of the sample are compared to DNA sequences from known species. Data from known species are available through online repositories such as the National Center for Biotechnology Information (NCBI). The methodology is easy and reliable, and it only requires a small sample of tissue to work. Most forms of visual identification require the entire organism. Because of these benefits,
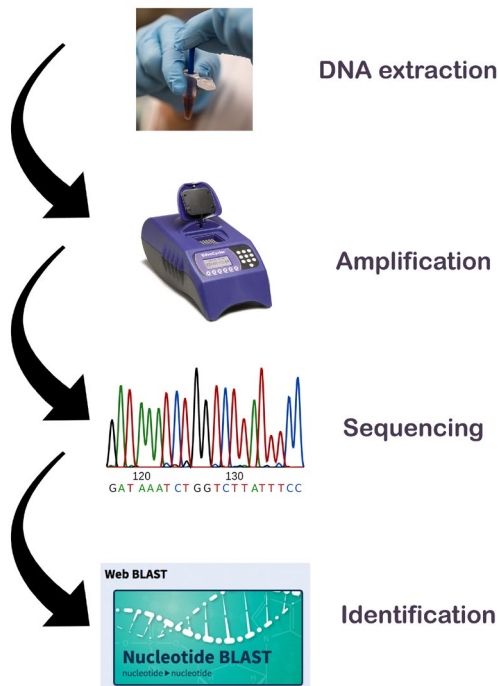

Figure 1. Standard workflow for the identification of an unknown species using DNA barcoding.

DNA barcoding has numerous biological applications including verification of disease vectors, preserving endangered species, monitoring water quality, and authentication of product ingredients such as food and medicines.

Fish Fraud in Louisiana

    In early 2018, enforcement agents from the Louisiana Department of Wildlife and Fisheries (LDWF) received a tip that a number of restaurants in central Louisiana were advertising "catfish" to their customers while actually selling Swai, which is a much cheaper fish native to Southeast Asia. On January 26th, after investigating the matter, LDWF ... LA)

RAPIDES PARISH, La. (LDWF) - Louisiana Department of Wildlife and Fisheries enforcement agents cited three restaurant managers on Jan. 26 in central Louisiana for allegedly selling swai fish as catfish.

Agents cited Robert Fant, 63, of Pineville, Mollie McDowell, 27, of Pineville, and Michael Rhodes, 39, of Pineville, for selling swai fish as catfish to their customers.

Agents received a tip that the Rosie Jo's restaurant in Alexandria and the Crazy Cajun restaurant in Tioga were selling swai fish as catfish. Agents performed an inspection of both restaurants and found that catfish was listed on the menu and found swai fish in the freezer. Agents cited McDowell and Fant as a result of these restaurant inspections.

Agents also learned that the same practice occurs at the Debarge's Crawfish restaurant in Alexandria and cited Rhodes.

Crazy Cajun (KALB)

Selling swai fish as catfish brings a $400 to $950 fine and up to 120 days in jail.

Figure 2. KALB news story describing episodes of fish fraud uncovered in central Louisiana.

    In this lab, you will be investigating how widespread a problem fish fraud is in central Louisiana. Your instructor has collected a series of fish samples from local business across the area, and you will perform a DNA barcoding analysis on the samples to see if they are actually the species they are advertised as being. The first week of this experiment you will extract DNA from your fish samples, complete PCR amplification on the COI gene, and prepare the PCR products to be sent out for DNA sequencing. The second week you will examine your returned sequence data and use an online data repository to verify the identity of your unknown tissue sample.

Week 1

Procedure 1: Preparing the PCR Tubes

    In this procedure you will prepare several PCR tubes with the solutions you will need to amplify your DNA during the PCR procedure. The benefit to preparing the tubes before you extract your sample DNA is that you can begin the PCR process quickly after your DNA is successfully extracted. For this procedure you will prepare 50 µL reactions in each tube. This means the total volume of all solutions adds up to 50 µL. See Table 1 for itemized list of what goes into each tube. Note that the ONETAQ master mix contains *Taq* Polymerase as well as dNTP's and reaction buffers.

    You will prepare one PCR tube for each fish sample and an additional PCR tube for the negative control. There is no positive control in this particular experiment, but successful DNA amplifications can become future positive controls for PCR (positive control = experiment that has already worked well).

Table 1. List of components and their corresponding volumes needed for PCR amplification.

Components	Volume
Forward primer	3 µL
Reverse primer	3 µL
ONETAQ master mix	25 µL
Molecular grade H_2O	19 µL
Extracted DNA from your sample	
Total	50 µL

General Rules:
1. Always wear gloves; you want to amplify DNA material from the fish, not your own cells!
2. Note how many reactions you will prepare before you even begin
3. Keep water, ONETAQ master mix, and primers on ice at all times
4. Each member of the group should do at least one sample; don't designate one person to do all the work because technically that person should then also get all the credit
5. Important: Use a fresh tip for each step - you do not want to contaminate your reagents with sample material or other reagents

Protocol:
1. Label the lid of a PCR tube (Fig. 3) with your group designation and the sample number. Example: A1, A = group designation, 1 = sample number, A0 = negative control (no DNA)
2. Add 19 µL of water to the PCR tube
3. Add 3 µL of the forward primer
4. Add 3 µL of the reverse primer
5. Add 25 µL of the ONETAQ master mix
6. Mix the sample by pipetting the solution up and down five times starting at two or three different locations in the tube
7. The polymerase will be quite foamy. Close the lid and tap the bottom of the tube on the bench a few times; you can also use your thumb as a spring: trap and quickly release your index finger so that the nail hits the bottom of the tube; do this a few times and all foam bubbles should disappear
8. Repeat steps 2 through 7 for all remaining samples as well as the negative control. (*note that the only difference between the tubes at this point should be the label on the lid*)
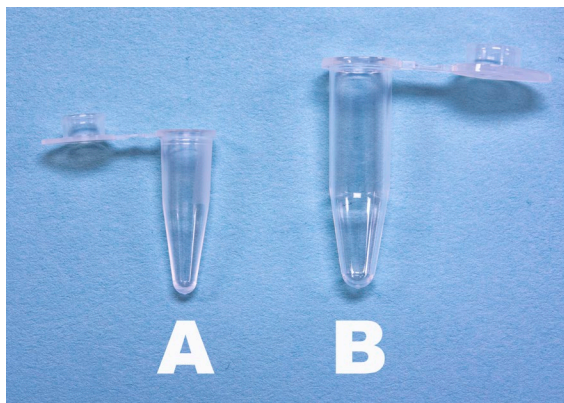
Figure 3. Two types of tubes commonly used in molecular research. A. 0.2mL PCR tube. B. 0.65 microcentrifuge tube.

Procedure 2: Extraction of DNA from Sample of Unknown Fish

In this portion of the lab you will use a novel DNA extraction method, referred to here as the "dipstick technique" to isolate DNA from your fish samples so that it can be used in the DNA barcoding process. The chemistry behind the process is simple: cellulose can trap or absorb DNA. Because of this, even something as common as a paper towel could theoretically be used to collect DNA. We will use Whatman filter papers because they are made from high-quality cotton sheets that have an alpha-cellulose content of at least 98%. The Whatman filter paper dipsticks were prepared by cutting the paper into 44 mm X 2 mm strips and coating a 40 mm length of those strips in wax. This waxy part is impervious to DNA and functions as a convenient handle so you can hold the dipstick without contaminating it with your own DNA. The "exposed" cellulose part of this dipstick is used to collect DNA from your sample tissue extraction. You will receive already prepared specimens of fish muscle tissue incubated over night in extraction

buffer at 55˚C. The higher tempratures help break down the muscle tissue.

Note: The fish samples you receive from your instructor will be suspended in a solution of extraction buffer. This buffer gets its name from the fact that it aids in the extraction of DNA from the tissues you are working with, but it contains small amounts of a chemical called SDS (Sodium dodecyl sulphate) which is considered a biohazardous reagent. SDS is harmful if swallowed, can cause serious eye irritation, and can cause mild skin irritation; so make sure you are always wearing gloves and be careful when handling any of these substances.

Protocol:
1. Obtain from your instructor a sample of unknown fish macerated overnight at 55˚C in extraction buffer
2. Use a homogenizer to gently grind the tissue by turning and twisting it around in the microcentrifuge tube for about 1 min
3. Vortex the sample for 20 seconds
4. Centrifuge the sample for about 20 more seconds at 10,000 rpm
5. Repeat steps 2, 3, and 4 until the tissue is thoroughly macerated and uniformly homogenized
6. Prepare a microcentrifuge tube with 200 µL of wash buffer
7. Take a dipstick at the waxed end and dip the bare end three times for about 1 sec into the macerated tissue
8. Dip the dipstick three times into the wash buffer, leaving it in the buffer for about 1 sec each time
9. Dip the dipstick three times into the PCR reaction tube you prepared in procedure 1
10. Repeat steps 1-8 for each fish sample; remember to prepare fresh wash buffer for each sample, otherwise your samples may be mixed and worthless
11. Repeat the same procedure for the negative control, but without adding a fish sample; instead, dip a fresh dipstick (without sample) three times into fresh extraction buffer, three times into fresh wash buffer, and three times into the negative control PCR tube, respectively
12. Close the PCR tubes' lids
13. Your instructor or TA will collect the tubes, load them into the PCR machine, and initiate the amplification process

Procedure 3: Amplification of the COI Gene Region

The thermocycler will now mediate the series of reactions that will amplify (replicate many times) the part of the COI gene region that is found between your forward and reverse primers. Your instructor has pre-programmed the machine to raise and lower the temperature of the tubes to follow these discrete steps:

Table 2. PCR amplification program.

Step	Temperature	Time	
Initial denaturation	94˚C	1 minute	
Denaturation	94˚C	40 seconds	
Annealing	53˚C	2 minutes	5 cycles
Elongation	68˚C	1 minute	
Denaturation	94˚C	30 seconds	
Annealing	58˚C	40 seconds	35 cycles
Elongation	68˚C	1 minute	
Elongation	68˚C	5 minutes	
Hold	4˚C	Until analysis	

Before the PCR program has completed make sure you prepare and label the tube that your instructor will send in for sequencing. Follow the guidelines below:
1. Label the lids of microcentrifuge tubes using the same notation as you did for the PCR tubes (the products inside each PCR tube will be transferred to their corresponding microcentrifuge tube)
2. Write your name and the date on the side of each tube in the rough part
3. Add the prescribed amount of primer to this tube (your instructor will tell you which primer and how much to use); important: you must only use one primer, you cannot use both
4. Close the lid and give the tube to your instructor

Post Lab Questions:

Describe three scenarios where species identification using DNA barcoding would be preferential to traditional means.

List the three stages of PCR and describe what happens in each stage.

What role does Taq polymerase play in this experiment? Investigate the history of Taq polymerase and describe it here.

Assuming you began with one copy of the COI gene, by the end of the PCR program you are using (see Table 2), how many copies will you have?

Week 2

GenBank is a genetic sequence database built and distributed by the National Center for Biotechnology Information (NCBI), which is located on the campus of the US National Institutes of Health (NIH; Benson et al, 2005). The NIH is devoted to medical research and consists of over 20 separate institutes and centers. The GenBank database is designed to provide the latest and most comprehensive DNA sequence information available within the scientific community. GenBank is part of the International Nucleotide Sequence Database Collaboration, which consists of the DNA DataBank of Japan, the European Nucleotide Archive, and GenBank. This comprehensive database contains annotated nucleotide sequences for more than 240,000 named organisms, and includes RNA segments, segments of genomic DNA, and entire genomes (NIH News Release, April 2008).

Each GenBank entry includes a scientific name, description of the sequence, some taxonomic information about the organism, references, and a table of features describing any areas of interest or significance. Every record in GenBank consists of both a sequence and its annotations. These records are given an identifier (accession number), which will remain constant over the lifetime of the record regardless if there is a change to the sequence or annotation (Benson et al, 2005).

Sequence searches are a regular part of characterizing gene functions, and also in identification of disease. Analyses of GenBank sequences have led to many important advances in science and medicine. In 1990 the Human Genome project was initiated, and as the data was generated it was submitted to GenBank. This 13-year project determined the sequences of the .

3 billion chemical base pairs that make up the human genome. GenBank also has many uses beyond human DNA. Scientists used GenBank to investigate colony collapse disorder in the honeybee and found a strong correlation to a specific virus (NIH News Release, April 2008).

Procedure 1: Using an Online Database to Match Your COI DNA Sequence to a Known Fish Species

Protocol for Sequence Selection:

1. You should have a free sequence viewer on your laptop; for the Mac we suggest 4Peaks (https://nucleobytes.com/4peaks/index.html) and for Windows we suggest Chromas (https://technelysium.com.au/wp/chromas/), but there are multiple options (free, professional, for Mac, Windows, and Linux systems
2. You will receive the sequence(s) of one or several species from your DNA extraction and PCR experiments in FASTA format; the first line starts with ">" followed by sequence identifiers in any possible form; the second and all following lines are the actual sequence; the very first line of a multiple FASTA format with several sequences starts with ";"
3. FASTA formats exist for nucleotide and amino acid data; we obtained DNA data from the PCR
4. Your sequence viewer shows red areas on both ends of the sequence; avoid using those, because sequencing quality diminishes close to the ends (see discussion)
5. You will see the usual four nucleotides (A, C, G, T), but also a few Ns. These represent positions in the DNA sequence for which the lab could not determine the actual nucleotide; therefore, an N could be any of the four (Fig. 4)
6. When highlighting the sequence to be sent in for identification, avoid Ns that will accumulate at the ends of the sequence
7. Some sequences will have very few Ns, some will have more; highlight a reasonably large stretch of sequence with as few Ns as possible, preferably without Ns
8. Your chosen sequence may be significantly shorter than the expected 648 nucleotides; ask your instructor or TA what sequence is appropriate for you to choose
9. Each app has a different way to send sequences to the NCBI (National Center for Biotechnology Information), usually involving "BLAST Sequence" and "Nucleotide (BLASTn)" commands; ask your instructor or TA to show you how to BLAST a sequence; if you would like to try it for yourself: in Chromas, click File and then scroll down to BLAST Search…; in the Window that opens, leave most of the standard settings, they will take you to the correct website; however, you must set your region (see Procedure 2 below) and tell the program what nucleotides you want to include in your search; don't start before nucleotide 50 and only use a region without Ns and pronounced peaks (Fig. 4); in 4Peaks, you have to first scroll and highlight the region you want to use; go to Edit and scroll down to BLAST sequence, then Nucleotide (BLASTn), and your sequence will be transferred directly to the NCBI
10. In a second round, choose the whole or almost the whole sequence that is not highlighted in red and ignore the Ns; compare the differences and discuss what they mean; discuss your findings with your instructor or TA
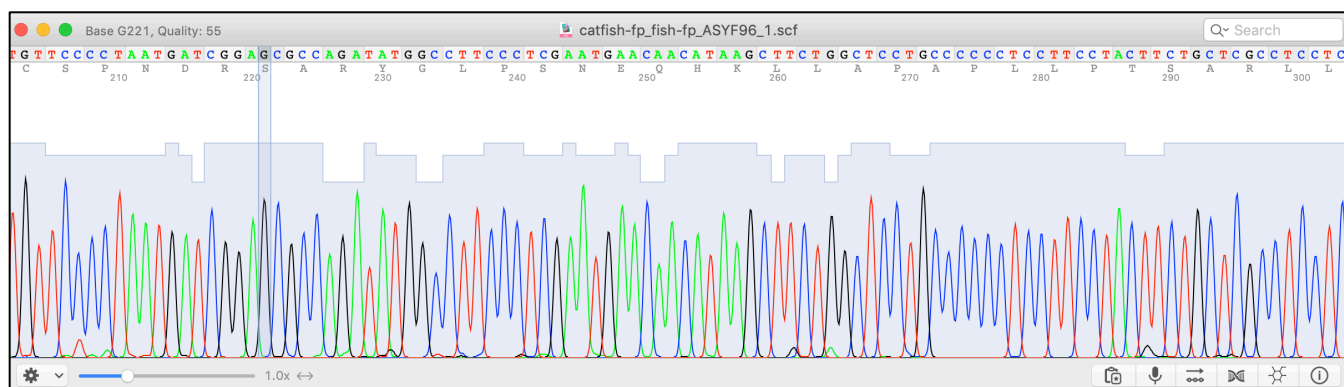

Figure 4. Screenshot of a sequence of the channel catfish, *Ictalurus punctatus*, in a sequence viewer program.

The sequence chromatogram shown in Fig. 4 is of excellent quality. The peaks are tall and there is very little overlap between each one, but you will more frequently see results that are not as pristine. Figure 5 highlights several additional examples of sequence chromatograms, each with a different degree of quality. Examine these so you can recognize in the future if you can use your results or if you will need to rerun the samples to get cleaner data. The chromatogram in Fig. 5A is of reasonable quality to be reliably used in an analysis. It would be considered "good." Figure 5B demonstrates a chromatogram

of low signal strength. You can determine this because the peaks are very short. There aren't a large number of unknown nucleotides (Ns), but the signal was still so weak that reliability is undermined. The chromatogram in Fig. 5C has quite a few Ns and there are a large number of overlapping peaks, which also undermine reliability. Figure 5D suffers from both low signal strength (short peaks) as well as overlapping peaks, making it the most unreliable source for sequence data.
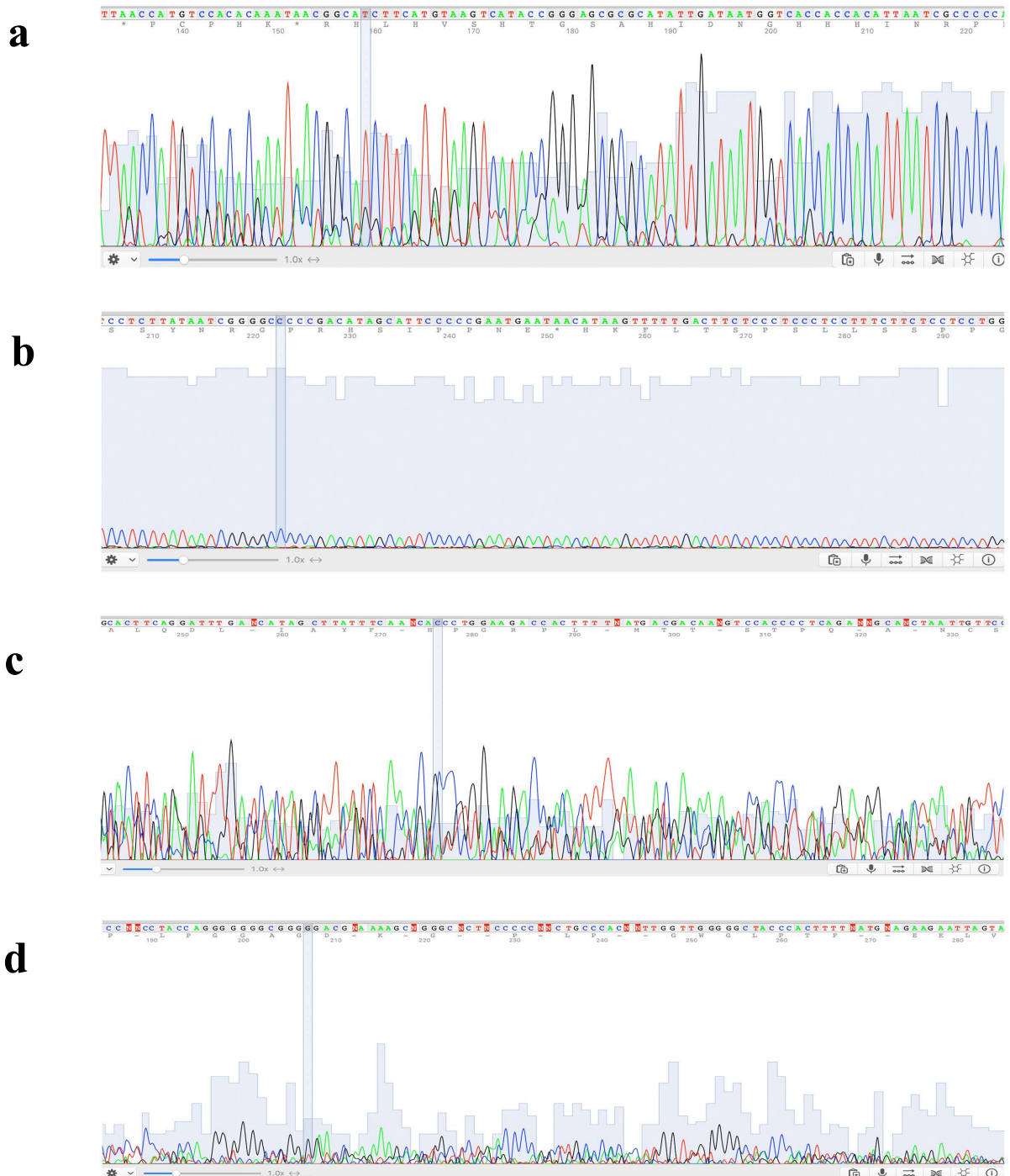
Figure 5. Four sequence chromatograms of varying quality. a) Crayfish (*Faxonella creaseri*); tall, minimally overlapping peaks with very few Ns (reliable). b) Channel catfish (*Ictalurus punctatus*); signal strength low (unreliable). c) Crayfish *(Faxonella creaseri)*; signals partially overlapping, many Ns (unreliable). d) Crayfish (*Faxonella creaseri*); low signal strength and several overlapping signals (unreliable).

Procedure 2: Bioinformatics Analysis:

DNA sequence data can be used to answer many types of questions, and they are widely used for identifications of species and individuals within a species. Automated DNA sequencers are able to determine the order of the four bases and generate a chromatogram. A chromatogram depicts the signal intensities in a graph using four colors, each color representing a base (Guanine = black; Cytosine = blue; Thymine = red; Adenine = green). The height of a peak corresponds to the concentration of that base, at that position in the sequence. When looking at a chromatogram you should see evenly spaced peaks, with only one color being visible for each peak. Variability (differences) in peak heights are normal, but you should be aware of baseline noise which shows up as peaks and may make it impossible to determine the real nucleotide. In addition, as the gel progresses you will see the peaks become more spaced apart, which is a sign of the gel losing resolution. At some point the spacing between the peaks will become irregular (wide peaks, multiple bases where there should only be one) and that is a sign that the chromatogram has become unreliable. Use the chromatograms provided by your instructor to familiarize yourself with good and unreliable chromatograms.

A good sequencing chromatogram should have well-resolved peaks with little to no background noise. You may also use the gray bars above each base to help you decide how reliable that sequence is at that particular point. The higher the bar, the more certain the program was when it identified that base. In a bad sequence chromatogram, the peaks may overlap and very few of the gray bars will be above the dotted green line (the gray bars will not be very high). In addition, most unreliable chromatograms will have background noise that will prevent you from being able to determine which peak is the nucleotide.

Even in a good chromatogram the first and last 25 to 50 base-calls are unreliable, and they should be deleted from your analysis (Fig. 4). This step is called trimming the ends of the chromatogram. When you trim the chromatogram, the actual peaks will not disappear but all the letters above the peaks will.

Sequence searches are the most frequent and simplest type of analysis performed on the GenBank database. These basic local alignment search tool (BLAST) searches allow a researcher to compare a query sequence with a database of sequences (Fig. 6). Each alignment returned by a BLAST search receives a score and a statistical significance, which indicates the quality of the search (Wheeler et al, 2005). This particular search is popular because it quickly identifies regions of similarity between two sequences. When you do a BLAST search, you will get a list of entries in GenBank that most closely match your sequence, with the best match at the top of the list (Fig. 7). Often there will be several entries for the same genus and species, especially if that species has been involved in numerous studies.

If you scroll down below the list of BLAST matches you will see the actual alignment of your sequence to the best match in GenBank. The Query sequence is the sequence you submitted in your search. The subject sequence is the sequence that is the best match to your sequence in GenBank. If you look at the identities value within the results you will see how similar your sequence is to the sequence in GenBank (Fig. 7). The numbers will indicate how many bases were the same in the submitted sequence, and the value found in the parentheses will tell you how similar the bases are in your submitted sequence on a percentage basis. The Expect value (e-value) of the hit is the match you can expect by chance, a smaller e-value indicates a better match.

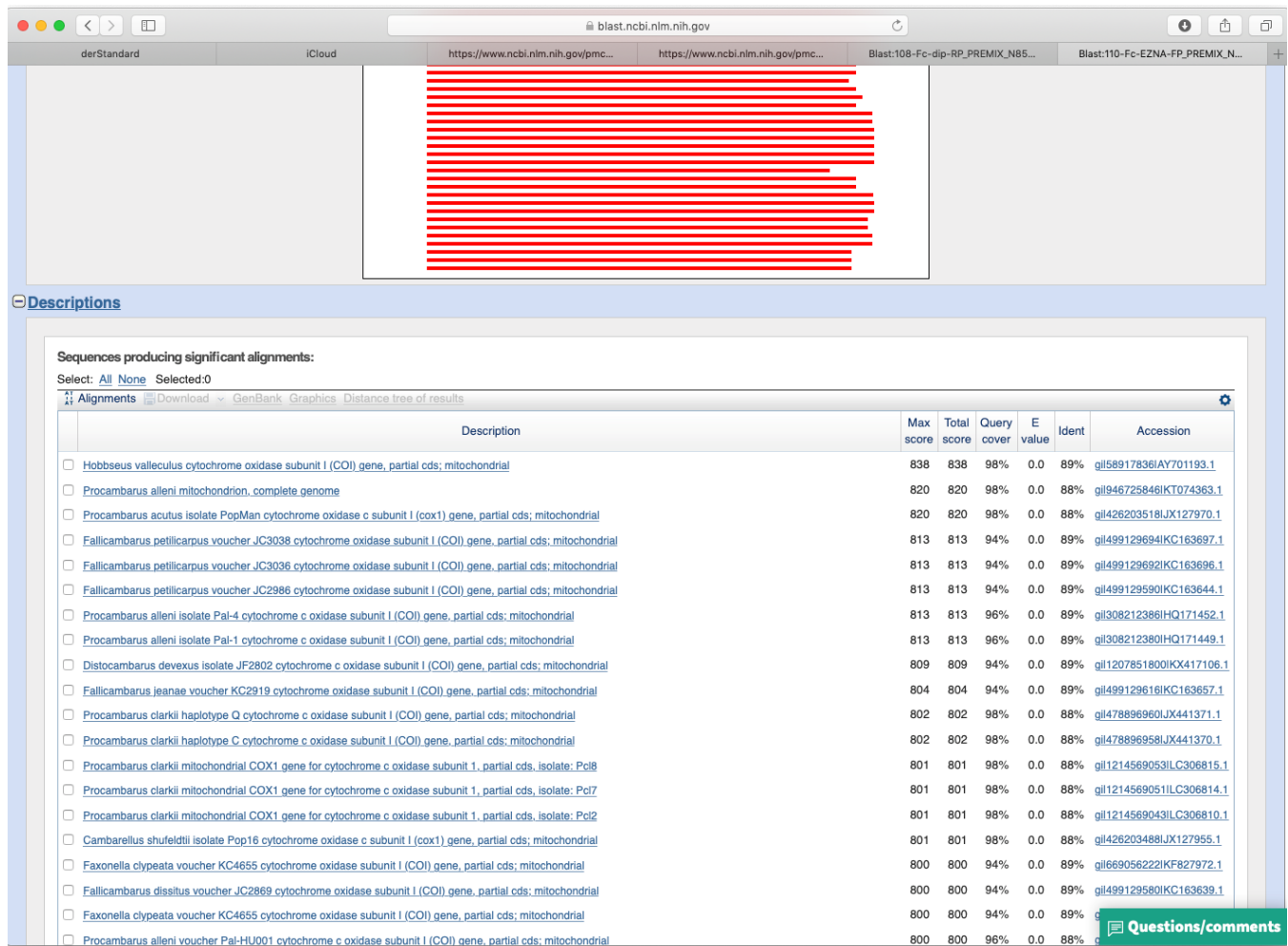**Figure 6**. Screenshot of the standard BLAST search page.

Figure 7. Screenshot of a BLAST search outcome for channel catfish, *Ictalurus punctatus*.

Protocol for Species Determination:

1. All the necessary information will have already been entered into the BLAST fields; we will accept the standard setup suggested by the website (Fig. 6)
2. Your instructor will discuss different BLAST search options with the class
3. Push the BLAST button at the bottom of the page
4. Depending on the sequence, time of day and year, and the speed of the internet connection sequence processing may take 10 sec to several minutes
5. The resulting page will show you the top hits with the closest sequence similarities (Fig. 7)
6. The graph shows these top hits with similarities portrayed in color
7. The specific sequences resulting in top hits are shown below the graph
8. The different sequences will be from different species; it is up to you to interpret the data; be cognizant of the fact that the species you are looking for my not be in the database; there is no predetermined level of similarity that tells you that this is the species you are looking for
9. You can rank the resulting sequences according to "Max score", "Total score", "Query cover", "E value", and "Ident"
10. Discuss your findings with your instructor or TA

<u>Some Terms to Know:</u>

E Value: expectation to find the same sequence by chance; the lower the better

Query Cover: percent of your sequence aligned to a sequence in the databank; the higher the better

Ident: percent nucleotides identical with the reference sequence; the higher the better; a visual comparison of sequences is shown below the list of species names

Max Score: highest alignment score of the best aligned sequence; should be equal to Total score, if there is only one sequence (as in our example); criteria for calculation include match rewards and mismatch penalties; usually results in the same ranking as e value

Write your lab report according to the criteria given to you by your instructor or TA.

Cited References

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Materials

Fish:

- Five different species of fish (from any source such as grocery store, fish market, etc.); keep refrigerated until use; frozen pieces need to be thawed in refrigerator overnight, fresh fish should be filleted; each group receives samples from all five species; prepare fish by weighing 20 mg muscle tissue for each laboratory specimen for each group; cut into pieces and place into microcentrifuge tube; add 400 µL extraction buffer and incubate over night at 55°C
- Five scalpels or sharp knives to cut fish
- Ethanol for sterilization of scalpels

Dipsticks:

To make dipsticks, cut Whatman No. 1 filter paper into 44 x 2 mm strips; dip them in wax up to 40 mm leaving a 4 mm space for the DNA to adhere to; produce 100 dipsticks, just to be sure; we had great success with borrowed histology wax equipment; two persons can do the work in 2 hr

- Pack of Whatman No. 1 filter paper
- Paraplast X-TRA tissue embedding medium; heat wax at ca. 55°C (in a miniature oven like the ones used in histology, or a heat chamber like the ones used in microbiology, or a water bath, or in the microwave)

DNA Extraction:

- Molecular grade H_2O; for biological barcoding, deionized and autoclaved tap water will work
- TRIS pH 8.0, 1M
- SDS
- Tween 20
- NaCl, 5M
- Extraction buffer: 0.5M NaCl, 1% SDS; for molecular biology labs, a stock solution of 10% (w/v) SDS is common; for 50 mL extraction buffer, use 5 mL 10% SDS, 5 mL 5M NaCl, and 40 mL H_2O
- Wash buffer: 10 mM Tris, pH 8.0, 0.1% Tween-20; for 25 mL of wash buffer, use 2.5 mL 1M TRIS, pH 8.0, 25 µL Tween 20, and 22.5 mL H_2O; ignore the additional volume
- Prepare 50 mL of extraction buffer and 25 mL of wash buffer and distribute equally to the five stations
- Ten 50 mL beakers or other glassware for extraction buffer and wash buffer for the five stations; autoclave before use

- Five vortexes, one for each station; students may also share fewer vortexes at a central location
- Five centrifuges, one for each station; students may also share fewer centrifuges at a central location
- Pellet pestles 1.5 mL, autoclavable, pack of 100; autoclave before use
- Pack of microcentrifuge tubes, autoclaved
- Five racks for microcentrifuge tubes
- Scales to weigh 20 mg tissue; not necessary, if fish is provided already cut and weighed
- Five pipette racks with five times the following pipettes: 1000 µL, 1-200 µL, 0.5-10 µL
- Autoclaved 1000 µL tips, five racks
- 1-200 µL extended filter tips, sterile, five racks
- 0.1-10 µL extended filter tips, sterile, five racks
- Gloves, three or four sizes (S, M, L, XL)

Recommended:

- Polyethylene foam to cover work area on bench

PCR:

- One PCR thermal cycler
- PCR tubes; one pack of 1000
- Five insulate ice containers
- Sharpie® Fine Tip Permanent Marker, 2 dozen
- ONETAQ® QUICK-LOAD® 2X Master Mix with Standard Buffer, NEB, 500 reactions
- Molecular grade water or deionized and autoclaved water; fill into ten microcentrifuge tubes for use and distribute to stations
- Primers: order FishF1, forward primer, and FishR1, reverse primer, 0.2 mM each for longtime use and dilute to 10 nM for amplification; at each of the five stations, add microcentrifuge tubes with 100 µL of 10 nM forward and reverse primers, respectively;
  FishF1
  5' - TCAACCAACCACAAAGACATTGGCAC -3'
  FishR1
  5' - TAGACTTCTGGGTGGCCAAAGAATCA -3'
  (Ward et al., 2005)

Analysis:

Each student has to have his/her own laptop with working internet connection. For Windows, Chromas (https://technelysium.com.au/wp/chromas/), and for the Mac, 4Peaks (https://nucleobytes.com/4peaks/index.html) should be uploaded.

Notes for the Instructor

Students are expected to work in groups of five. Each group needs to have access to at least one personal laptop or computer lab station for the analysis of sequences. Suggested software needs to be downloaded before the analysis lab. The lab handout needs to be read before lab.

Students should already be familiar with the concept of PCR from a previous lab or lecture - or it must be discussed in a separate session with the instructor. The excellent animation from Cold Spring Harbor Laboratory, https://www.dnalc.org/resources/animations/pcr.html, can be shown for demonstration purposes. Familiarize yourself with all procedures. Demonstrate the whole setup for each step for the DNA extraction and PCR procedures.

Prepare a sheet with notes for each group. Each student will prepare one microcentrifuge tube with either reverse or forward primer to which you will add his/her DNA template from the PCR. Standard volumes are 10 µL template and 5 µL primer. Each fish should be sequenced five times, preferentially either twice with fp and thrice with rp, or twice with rp and thrice with fp.

After the PCR finishes, add as much template as your favorite DNA sequencing facility wants you to add to the prepared student microcentrifuge tubes for sequencing. If your in-house sequencing facility prefers other methods of turning in samples, please follow their requirements.

During the analysis lab, demonstrate on a computer screen how students have to highlight and send off their sequences to NCBI using the apps that they downloaded. Discuss results and what they mean with the students. If possible, show examples of previous biological barcoding experiments. Explain how students have to write their lab reports.

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Acknowledgments

About the Authors

Christof Stumpf is a Professor of Biology at LSUA, Nathan Sammons is an Associate Professors of Biology at LSUA, and Susan Bowers is an Assistant Professor of Biology at LSUA. Stumpf and Sammons teach Introductory Biology and Evolution; their specialties are insects and other invertebrates (Stumpf) and plants (Sammons). Bowers is an Animal Physiologist and has experience in molecular biology. Stumpf started research in biological barcoding and Sammons started to reorganize the BIOL 1201 labs. Both agreed that working with DNA should be part of this course, which eventually led to the birth of this new lab activity. Bowers has taken courses in bioinformatics and will include the new procedure in her human Anatomy and Physiology courses and also in her Animal Physiology lab course.

Appendix A: Sample Results

After originally running this lab with "universal" primers, we switched to specific fish primers (Ward et al., 2005). With the new perimers, we ran PCR for both channel catfish (Fig. 5) and Atlantic salmon (Fig. 6) from a test run in December 2018 and from the Introductory Biology course in spring 2019. Because we have not been able to send in PCR from student experiments for sequencing with fish primers at this point, we refer here to the test results from 2018. The fish primers lead to clear species identifications. However, there were some differences between sequences. For the catfish, we could choose almost the whole sequence for comparison with the database with the sequence viewer revealing relatively large peaks without overlap (Fig. 3), leading to 97% query cover and 97.5% identification (Fig. 5). The larger the query cover, the lower the chance of 100% identity, but there was still only channel catfish available as a choice from the database. For the Atlantic salmon, peaks were smaller (Fig. 9) and we had to choose a much smaller part of the sequence for comparison with the database. As a result, we had 100% query coverage and 100% sequence similarity (Fig. 8), but for a shorter sequence. It is possible to have 100% identity with the wrong species, if the chosen sequence is too short, but we are certain that was not the case here. Results all depend on the quality of the amplified DNA samples. Therefore, careful analysis is always necessary, and there are quite a few pitfalls in biological barcoding. Examples like these should be discussed with students.
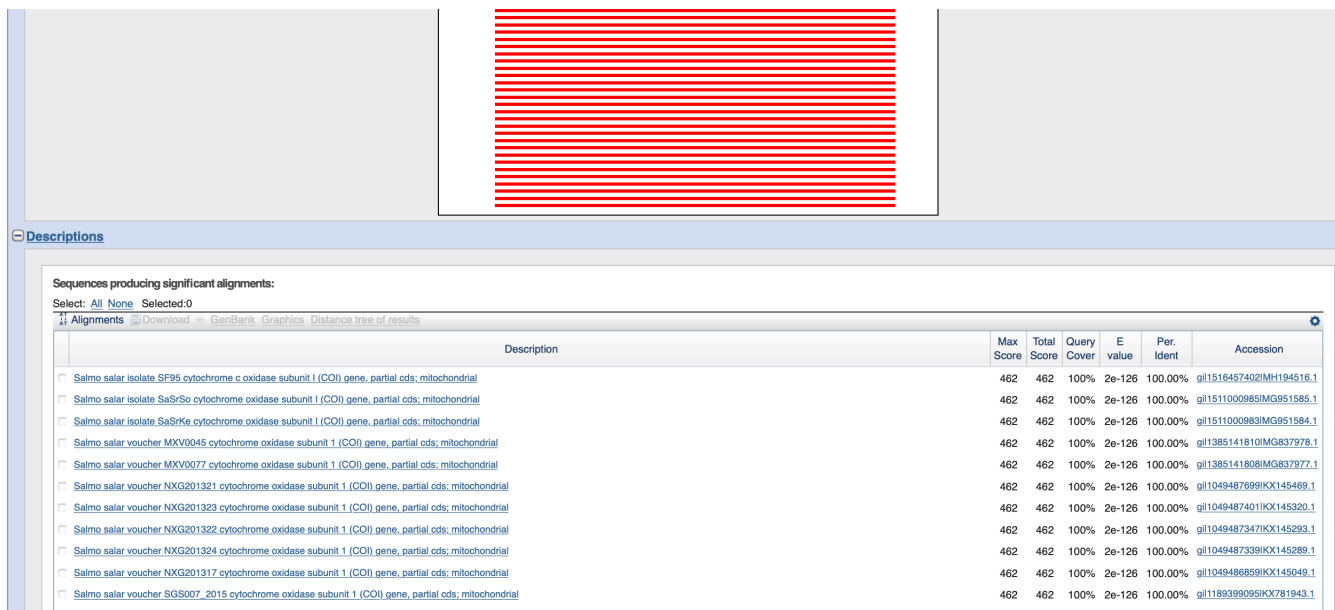

Descriptions

Sequences producing significant alignments:

Select: All None   Selected:0

Alignments   Download   GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Salmo salar isolate SF95 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1516457402IMH194516.1
Salmo salar isolate SaSrSo cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1511000985IMG951585.1
Salmo salar isolate SaSrKe cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1511000983IMG951584.1
Salmo salar voucher MXV0045 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1385141810IMG837978.1
Salmo salar voucher MXV0077 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1385141808IMG837977.1
Salmo salar voucher NXG201321 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1049487699IKX145469.1
Salmo salar voucher NXG201323 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1049487401IKX145320.1
Salmo salar voucher NXG201322 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1049487347IKX145293.1
Salmo salar voucher NXG201324 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1049487339IKX145289.1
Salmo salar voucher NXG201317 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1049486859IKX145049.1
Salmo salar voucher SGS007_2015 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial	462	462	100%	2e-126	100.00%	gil1189399095IKX781943.1

Figure 8. Screenshot of a BLAST search outcome for Atlantic salmon, *Salmo salar*.
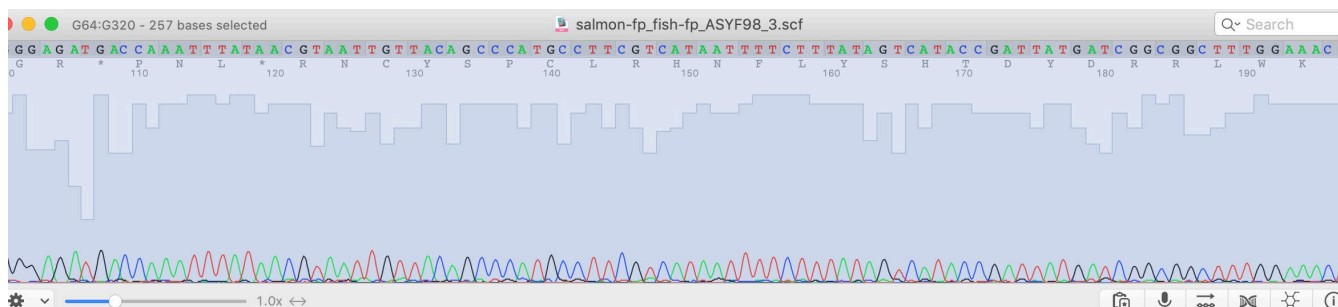

Figure 9. Screenshot of a sequence of the Atlantic salmon, *Salmo salar*, in a sequence viewer program.

Description of Laboratory Presentation:

At the ABLE conference, the workshop participants would directly experience all the important stages of this lab, including:

Preparation of DNA Extraction Papers

Participants would experience, first-hand, preparation of the DNA extraction strips by cutting the provided Watman paper into strips and coating them in wax, just as they would prior to the execution of the lab with the students.

DNA Extraction

Participants would go through all of the DNA extraction protocols and begin the PCR process. If any participants would like, we can also review how to program a PCR machine, or how to locate a facility close to their home institution that can sequence their students' fish DNA samples.

Analysis

We will provide sample chromatograms for the participants to use so that they may experience the database portion of the workshop. They will then perform an NCBI search to identify the species of their unknowns.

Mission, Review Process & Disclaimer

The Association for Biology Laboratory Education (ABLE) was founded in 1979 to promote information exchange among university and college educators actively concerned with teaching biology in a laboratory setting. The focus of ABLE is to improve the undergraduate biology laboratory experience by promoting the development and dissemination of interesting, innovative, and reliable laboratory exercises. For more information about ABLE, please visit **http://www.ableweb.org/.**

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