Investigation
21

Introduction to Molecular Evolution

OVERVIEW

An evolutionary tree shows the evolutionary lineages of different species over relative time. Evolutionary trees, also called *cladograms*, can be based on many different types of data. The traditional way of constructing evolutionary trees was to look at the physical morphology of organisms, including sizes, shapes and developmental structures of both living organisms and fossils. Today, similarities and differences in molecular data (protein and DNA sequences) are also being used. Both methods are valuable and often complement each other.

Your students will use the evolutionary tree in Figure 1 of the Preliminary Activity to make predictions about the relatedness of the species you will examine in this investigation. Following the analysis and interpretation of the electrophoresis results, your students will create a cladogram from their results and compare their cladogram with their predictions.

In the Preliminary Activity, your students will (1) extract proteins from the muscle tissue of catfish, salmon and shark, (2) conduct electrophoresis of the resulting protein extracts, (3) analyze the results using Logger *Pro*, and (4) construct a cladogram using the results. A student handout for the Open Inquiry version of the Preliminary Activity can be found at the end of this investigation. A Guided Inquiry version can be found on the CD accompanying this book.

During the subsequent Inquiry Process, your students will first find out more about protein gel electrophoresis using the course textbook, other available books, and the Internet. They will then generate and investigate researchable questions. (In the Guided Inquiry approach, students will plan and conduct investigations of the researchable question(s) assigned by you.)

See the student handout for an introduction to this investigation. For more thorough background information, see the Background section on pages 6–25 of the Instructor's Manual accompanying the "Comparative Proteomics Kit I: Protein Profiler Module." (Bio-Rad Laboratories, Inc., explorer.bio-rad.com, Catalog # 166-2700EDU)

LEARNING OUTCOMES

In this inquiry investigation, students will

- Identify variables, design and perform the investigation, collect data, analyze data, draw a conclusion, and formulate a knowledge claim based on evidence from the investigation.
- Learn protein extraction techniques.
- Perform gel electrophoresis on proteins.
- Investigate the evolution muscle proteins.
- Establish phylogenetic relationships based on molecular data.
- Use molecular data to create cladograms.

THE INQUIRY PROCESS

Suggested Time to Complete the Investigation

See page xiii in the Doing Inquiry Investigations section for more information on carrying out each phase of an inquiry investigation.

	Inquiry Phase	Open Inquiry	Guided Inquiry
I	Preliminary Activity	150 minutes	150 minutes
II	Generating Researchable Questions (Omitted in Guided Inquiry Approach)	10 minutes	0 minutes
III	Planning	10 minutes	10 minutes
IV	Carrying Out the Plan	120 minutes	120 minutes
V	Organizing the Data	10 minutes	10 minutes
VI	Communicating the Results	10 minutes	10 minutes
VII	Conclusion	5 minutes	5 minutes

MATERIALS

Make the following materials available for student use. Items in bold are needed for the Preliminary Activity.

Part I Protein Extraction from Muscle

three 1.5 mL flip-top microtubes
three 1.5 mL screwcap microtubes
1 mL transfer pipet
fish samples, labeled 1–3
marking pen
Laemmli sample buffer
knife or scissors to cut fish samples
water bath set to 95°C
others as requested by students

Part II Electrophoresis: Gel Loading, Running, and Staining

fish protein extracts from Part 1
actin and myosin standard
Precision Plus Protein™ Kaleidoscope™ prestained standards
4–20% Mini-PROTEAN® TGX™ gels
1–20 µL adjustable-volume micropipet*
Prot/Elec™ pipet tips for gel loading
Mini-PROTEAN Tetra cell electrophoresis module
1x Tris-glycine-SDS (TGS) buffer
power supply (200 V constant)**
sample loading guide for 10-well comb (optional)
buffer dam***
staining tray
Bio-Safe™ Coomassie stain
water bath set to 95°C
water for gel destaining
others as requested by students

* 5 µL and 10 µL fixed-volume pipets may be substituted
** Power supplies may be shared between work stations
*** Only required if running one gel per box

Part III Analysis

computer
Logger *Pro*
Vernier White Digital Bioimaging System
others as requested by students

I Preliminary Activity

This inquiry begins with an activity to reinforce prior knowledge of the use of Vernier technology and to introduce gel electrophoresis of proteins.

Sample Results

Additional sample data tables and gel images can be found in the Preliminary Activity. Student results may vary if different species were used.

Table 1: Number of Bands in Common			
	Shark	Catfish	Salmon
Shark	**7**	3	1
Catfish	3	**6**	3
Salmon	1	3	**9**

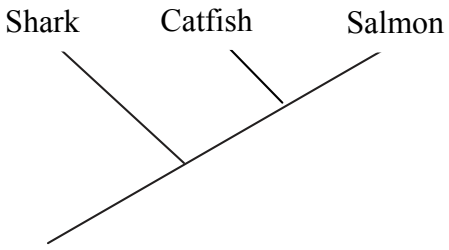

Figure 1 *Cladogram based on example data from the Preliminary Activity*

Answers to the Questions

1. Compare your cladogram with your original predictions.

   Answers may vary. The shark is an ancestral species, so it has a node at the base of the cladogram. The catfish shares three bands in common with the shark and the salmon, therefore the node for the catfish is placed in the middle of the cladogram. The salmon has only one band in common with the shark, and three bands in common with the catfish. As a result, the salmon is located at the top of the cladogram.

2. What was the purpose of heating the samples?

   The heat helps to denature the protein tertiary and quaternary structures, so that the proteins become less three dimensional and more linear.

3. How were the proteins extracted from the fish samples?

   The Laemmli sample buffer contains detergent that breaks open the cell membranes of the muscle cells. By flicking the tubes, the muscle tissue was also mechanically disrupted. These two things released proteins from the muscle tissue.

4. Why do SDS-coated proteins move when placed in an electric field?

   SDS is a negatively charged molecule that sticks to the polypeptide chain and adds a negative charge to the protein that is proportional to its length. The SDS-coated proteins move away from the negative charge towards the positive charge.

5. What is the purpose of the actin and myosin standards and the Precision Plus Protein Kaleidoscope prestained standard?

   The actin and myosin standard contains two common muscle proteins that have already been isolated. They serve as a good reference to locate actin and myosin proteins in the samples and act as a positive control for gel analysis. The proteins in the Precision Plus Protein Kaleidoscope prestained standard have known molecular weights. The prestained standard provides a size reference so that a standard curve can be drawn. Sample proteins are then compared to this standard curve to calculate the molecular weights of the unknown proteins in the muscle samples.

6. What is the purpose of the stain?

   Proteins are invisible in the gel. The stain contains a dye that sticks to the proteins and makes them visible.

7. List at least three common sources of the proteins actin and myosin, in addition to fish muscle.

   Some common sources of the proteins actin and myosin in addition to fish muscle are chicken, duck, goose, turkey, pig, cow, and sheep muscle.

8. List at least one researchable question dealing with gel electrophoresis of proteins.

   Answers will vary. See the Researchable Questions list below for some possible answers.

II Generating Researchable Questions

Note: Researchable questions are assigned by the instructor in the Guided Inquiry approach. See page xiii in the Doing Inquiry Investigations section for a list of suggestions for generating researchable questions. Some possible researchable questions for this investigation are listed below:

Recommended for Open Inquiry or Guided Inquiry (sample results provided)

- How do the muscle proteins of shark, catfish, and two salmonids (salmon and trout) compare?
- How do the muscle proteins of shark, sturgeon, mahi-mahi, and marlin compare?

Recommended for Open Inquiry or Guided Inquiry (no sample results provided)

- How do the muscle proteins of shark, sturgeon, catfish, and salmon compare?
- How do the muscle proteins of shark, sturgeon, catfish, and tuna compare?
- How do the muscle proteins of salmon, chicken, and pork compare?

Recommended for Advanced Students (sample results provided)

- How do the muscle proteins of shark, catfish, and two different salmon compare?
- How do the muscle proteins of shark, catfish, and three different salmonids compare?

Recommended for Advanced Students (no sample results provided)

- How do the muscle proteins of shark, sturgeon, catfish, salmon, and trout compare?

- How do the muscle proteins of shark, sturgeon, catfish, salmon, and tuna compare?
- How do the muscle proteins of shark, catfish, and different species of tuna compare?
- How do the muscle proteins of shark, sturgeon, catfish, trout, tilapia, and tuna compare?
- How do the muscle proteins of shark, salmon, frog, chicken, and pork compare?

There are many more possible researchable questions. Students should choose a researchable question that addresses the learning outcomes of your specific standards. Be sure to emphasize experimental control and variables. (Instructors using the Guided Inquiry approach select the researchable questions to be investigated by their students. We encourage you to assign multiple researchable questions because this strategy enhances student interaction and learning during phases IV–VII.)

III Planning

During this phase students should formulate a hypothesis, determine the experimental design and setup, and write a method they will use to collect data. The plan should list laboratory safety concerns and specify how they will be addressed during the investigation. Circulate among the student groups asking questions and making helpful suggestions.

IV Carrying Out the Plan

During this phase, students use their plan to carry out the investigation and collect data. Circulate among the student groups asking questions and making helpful suggestions.

V Organizing the Data

See page xv in the Doing Inquiry Investigations section for suggestions concerning how students can organize their data for their inquiry presentations.

VI Communicating the Results

See page xv in the Doing Inquiry Investigations section for a list of inquiry-presentation strategies.

VII Conclusion

Using your notes recorded during the Communicating the Results phase, summarize the group results for the experiment and tell how they will fit into the upcoming instruction.

VIII Assessment

See page xv in the Doing Inquiry Investigations section for ideas on assessment strategies.

SAMPLE RESULTS

Comparing Protein Similarities between Shark, Catfish and Salmonids

Table 2: Number of Bands in Common for Shark, Catfish, Salmon and Trout				
	Shark	Catfish	Salmon	Trout
Shark	**7**	3	1	1
Catfish	3	**6**	3	3
Salmon	1	3	**9**	**8**
Trout	1	3	**8**	**12**

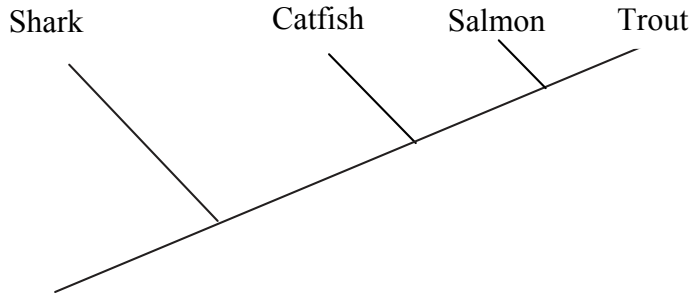

Figure 1 *Cladogram for shark, catfish, and two salmonids*

This investigation addresses the question, "How do the muscle proteins of shark, catfish, salmon, and trout compare?" Muscle banding patterns were determined using the Preliminary Activity procedure. The shark is an ancestral fish species since it has the fewest bands in common with the three other fish. As a result, the node for the shark is placed at the base of the cladogram. The catfish shares three bands in common with the shark and three bands in common with the salmonids. As a result, it is placed in an intermediate position in the cladogram. The salmon and the trout belong to the same family, the salmonidae. These two fish share eight bands in common with each other. As a result, the salmonids are placed at the top of the cladogram. The salmon has a total of nine bands and the trout has a total of twelve bands. The trout has three more bands than the salmon which suggests that it is more derived. As a result, the trout is located at the top of the cladogram.

Comparing Protein Similarities between Shark, Sturgeon, Marlin and Mahi-mahi

Table 3: Number of Bands in Common for Shark, Sturgeon, Marlin and Mahi-mahi				
	Shark	Marlin	Mahi-mahi	Sturgeon
Shark	**6**	2	1	3
Marlin	2	**6**	5	1
Mahi-mahi	1	5	**7**	**2**
Sturgeon	3	4	**5**	**8**

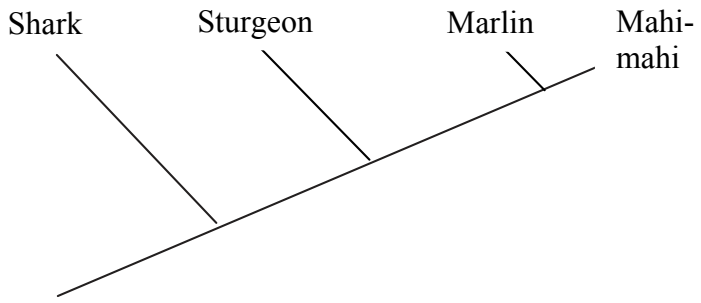

Figure 2 *Cladogram for shark, sturgeon, marlin, and mahi-mahi*

This investigation addresses the question, "How do the muscle proteins of shark, sturgeon, marlin and mahi-mahi compare?" Muscle banding patterns were determined using the Preliminary Activity procedure. The shark is an ancestral fish species since it has the fewest bands in common with the three other fish. As a result, the node for the shark is placed at the base of the cladogram. The sturgeon shares three bands in common with the shark, two bands in common with the mahi-mahi and one band in common with the marlin. As a result, the sturgeon is placed in an intermediate position in the cladogram. The mahi-mahi and the marlin belong to the same order, perciformes. These two fish share five bands in common with each other. As a result, these two fish are placed together at the top of the cladogram. The marlin has a total of six bands and the mahi-mahi has a total of seven bands. The mahi-mahi has one more band than the marlin. This suggests that the mahi-mahi is more derived. As a result, the mahi-mahi is located at the top of the cladogram.

Comparing Protein Similarities between Shark, Sturgeon, Salmon species

Table 4: Number of Bands in Common for Shark, Catfish, and Two Species of Salmon				
	Shark	Catfish	Atlantic salmon	Chinook salmon
Shark	**7**	3	1	1
Catfish	3	**6**	3	3
Atlantic Salmon	1	3	**9**	**8**
Chinook Salmon	1	3	**8**	**9**

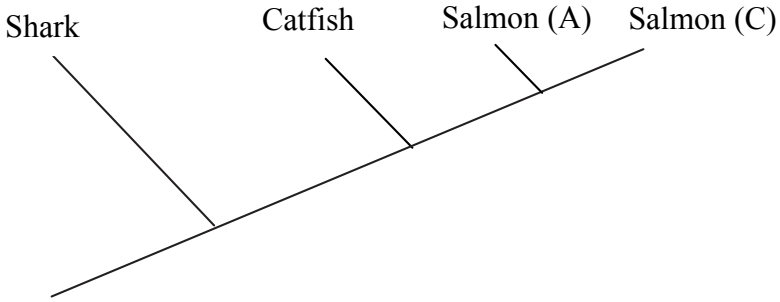

Figure 3 *Cladogram for shark, catfish, Atlantic salmon and Chinook salmon*

This investigation addresses the question, "How do the muscle proteins of shark, catfish, Atlantic salmon and Chinook salmon compare?" Muscle banding patterns were determined using the Preliminary Activity procedure. The shark is an ancestral fish species since it has the fewest bands in common with the three other fish. As a result, the node for the shark is placed at the base of the cladogram. The catfish shares three bands in common with the shark and three bands in common with the two salmon. As a result, it is placed in an intermediate position in the cladogram. The Atlantic salmon and the Chinook salmon belong to the same family, salmonidae. However, the salmon are not in the same genus. The Atlantic salmon is in the genus *Salmo*, while the Chinook salmon is in the genus *Onocorhyncus*. These two fish share eight bands in common with each other. As a result, these two fish are placed together at the top of the cladogram. The Chinook and the Atlantic salmon both have a total of nine bands. As a result, we cannot determine which species is more derived. We have placed the Chinook salmon at the top of the cladogram.

Comparing Protein Similarities between Shark, Sturgeon, and Three Salmonids

Table 5: Number of Bands in Common for Shark, Catfish, Atlantic Salmon, Chinook Salmon, and Trout

	Shark	Catfish	Atlantic salmon	Chinook salmon	Rainbow trout
Shark	**7**	3	1	1	1
Catfish	3	**6**	3	3	3
Atlantic Salmon	1	3	**9**	**8**	**8**
Chinook Salmon	1	3	**8**	**9**	**8**
Trout	1	3	**8**	**8**	**12**

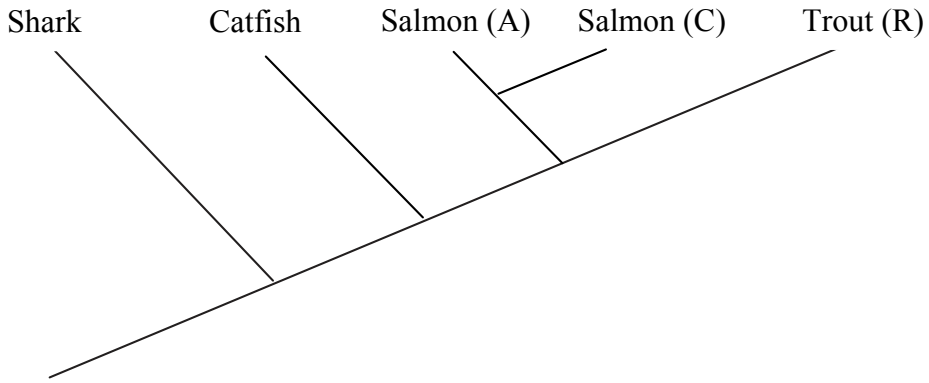

Figure 4 *Cladogram for shark, catfish, and three salmonids*

This investigation addresses the question, "How do the muscle proteins of shark, catfish, Atlantic salmon, Chinook salmon and rainbow trout compare?" Muscle banding patterns were determined using the Preliminary Activity procedure. The shark is an ancestral species since it has the fewest bands in common with the other fish. As a result, the node for the shark is placed at the base of the cladogram. The catfish shares three bands in common with the shark, and three bands in common with the salmonids. As a result, it is placed in an intermediate position in the cladogram. The Atlantic and Chinook salmon and the rainbow trout all belong to the same family, salmonidae. However, these three fish do not all share the same genus. The Atlantic salmon is in the genus *Salmo*, while the Chinook salmon and the rainbow trout are in the genus *Onocorhyncus*. All of these fish share eight bands in common with each other. As a result, these fish are placed at the top of the cladogram. The Chinook and the Atlantic salmon both have a total of nine bands, eight of which are shared. As a result, the two salmon species are placed together with a new node for the Chinook salmon. The trout has three more bands than the two salmon species which suggests that it is more derived. As a result, the trout is located at the top of the cladogram.

TIPS

1. The instructions provided assume that you have purchased the Bio-Rad kit, "Comparative Proteomics Kit I: Protein Profiler Module." (Bio-Rad Laboratories, Inc., explorer.bio-rad.com, Catalog # 166-2700EDU)

2. This Investigation can only be completed using computers.

3. When purchasing fish, keep in mind that you only need a small amount of each sample. At the fresh fish counter at your grocery store, you can probably get small samples for minimal or no charge. Frozen fish works just as well as fresh. Be sure to keep track of which fish is which.

4. Analysis of the gel is completed using the Vernier White Digital Bioimaging System connected to a computer running Logger *Pro* data-collection software. From the digital image of the gel, Logger *Pro* will create a standard curve and automatically calculate the molecular weight for each experimental protein band.

5. The Bio-Rad kit includes 0.3 grams of DTT (dithiothreitol) for optional use. DTT is sometimes used to ensure the complete breakage of disulfide bonds. It is, however, hazardous when inhaled, swallowed, or in contact with skin, and we recommend that you not use it for this investigation.

6. A complete list of materials for Part I of the Preliminary Activity is found in the Materials section. The materials listed here, **one per student group**, require special preparation:

   Fish samples sets: Cut each fish sample into a roughly 0.5–1 cm square piece. Place it on a card or a piece of plastic.

   Laemmli sample buffer: Aliquot 1.5 mL of Laemmli sample buffer into a 1.5 mL flip-top microtube and label it **SB**, for sample buffer. Store at room temperature.

7. A complete list of materials for Part II of the Preliminary Activity is found in the Materials section. The materials listed here, **one per student group**, require special preparation:

   Actin and myosin standard: Label a 1.5 mL screw-cap tube **AM**, for actin and myosin. Aliquot 12.5 µL of rehydrated and preheated actin and myosin standard (see preparation for this in Step 8) into the tube. Store at –20°C.

   Precision Plus Protein Kaleidoscope prestained protein standard: Label a 1.5 mL flip-top tube **Stds**, for standards. Aliquot 6 µL of standard into the tube. Store at –20°C.

8. Directions for preparation of the Part II reagents:

   Rehydrated Actin and Myosin Standard: Add 500 µL of Laemmli sample buffer to the vial of actin and myosin standard and incubate at room temperature for 20–30 minutes. Transfer the rehydrated actin and myosin sample to a screw-cap tube and heat for 5 minutes at 95°C. Store at –20°C.

   Tris-Glycine-SDS (TGS) running buffer: To make 3 L of 1x TGS running buffer, mix 300 mL of 10x TGS with 2,700 mL of distilled water. Store at room temperature. One Mini-PROTEAN Tetra cell with two gels requires 700 mL of 1x TGS running buffer. One Mini-PROTEAN Tetra cell using the companion running module to run four gels requires 1.1 L of 1x TGS running buffer.

   You may want to prepare 1–2 L of extra 1x TGS buffer in case your gel boxes leak after assembly. If you do have a leak, the outer chamber of the gel box can be filled to above the

inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 1,200 mL of 1x TGS buffer per gel box and is a more convenient fix than reassembling the apparatus mid-lesson.

9. When using the Vernier White Digital Bioimaging System, be sure to use the power supply that shipped with the device. Using a wrong power supply could damage the system.

10. Tips for using Logger *Pro* to take a photo of the stained gel:
    - For the best photo of the gel banding patterns, you may want to adjust the camera settings before clicking Take Photo. Click Camera Settings in the Take Gel Photo dialog box, and then click Adjustments. From here, position the brightness slider between 10 and 25%, making sure that the Imaging Hood is placed over the system. Continue to adjust until the best results are obtained.
    - When performing a gel analysis, the sequence of events described in the procedure must be followed in order. It is not possible to go back and change parameters, with these exceptions:
      - Points placed on the photograph identifying experimental bands can be moved or deleted. Simply click Select Point, , then click on the point to be edited. Drag it with the cursor to move it or use the Delete key on your keyboard to delete.
      - Points for experimental bands can be added by clicking Add Lane, , selecting the appropriate lane, and then clicking on the band in the photograph.

11. **Safety Precautions:**

    Good laboratory practice should be followed while carrying out all aspects of any laboratory procedure. We recommend that students wear gloves and safety glasses while handling fish samples, polyacrylamide gels, protein stain, and the other reagents used in this exercise. Gloves not only protect students from exposure to the reagents, such as the blue protein stain, but also protect the samples and gels from unwanted contamination from your students' hands. Be sure that students wash their hands and benchtops after working with fish.

    The DTT (dithiothreitol) included in the kit is harmful when inhaled, swallowed, or in contact with skin. We do not recommend that you use it for this investigation.

    Please refer to the MSDS for more information on the safety assessment of the reagents in this kit. The MSDSs are also available at www.bio-rad.com. Please consult your local environmental health and safety regulations for proper disposal.

    Note: Students with known seafood allergies should avoid all contact with fish and/or shellfish samples.

12. We do not recommend using blind samples. One ancestral reference species (e.g., shark muscle) should always be used as a control.

13. The plans that your students submit for approval should list laboratory safety concerns, including chemical safety concerns, and specify how they will address these safety concerns during their investigations.

This investigation was adapted from Bio-Rad Laboratory's Comparative Proteomics Kit I: Protein Profiler Module. Text and figures are used with permission.