A FISHY FAMILY TREE

We know that catfish, tuna, and swordfish are all fish and that all three share an ancestor. But which two of these fish share a more recent ancestor? In other words, which two of these fish are most closely related?

To find out, examine the illustrations of these three fish and of their scales. Then fill out the data chart below.

Kind of Fish	**Tail Fin Shape** (fan shaped vs. lobed)	**Presence of Barbels** ("whiskers" present vs. absent)	**Scale Type** (cycloid, ctenoid, etc.)
Catfish			
Tuna			
Swordfish			

1. Which two fish do you think are most closely related?

2. Please explain your reasoning.

3. Label the evolutionary tree below to show your hypothesis.
(Write the name of one kind of fish in each of the three boxes.)

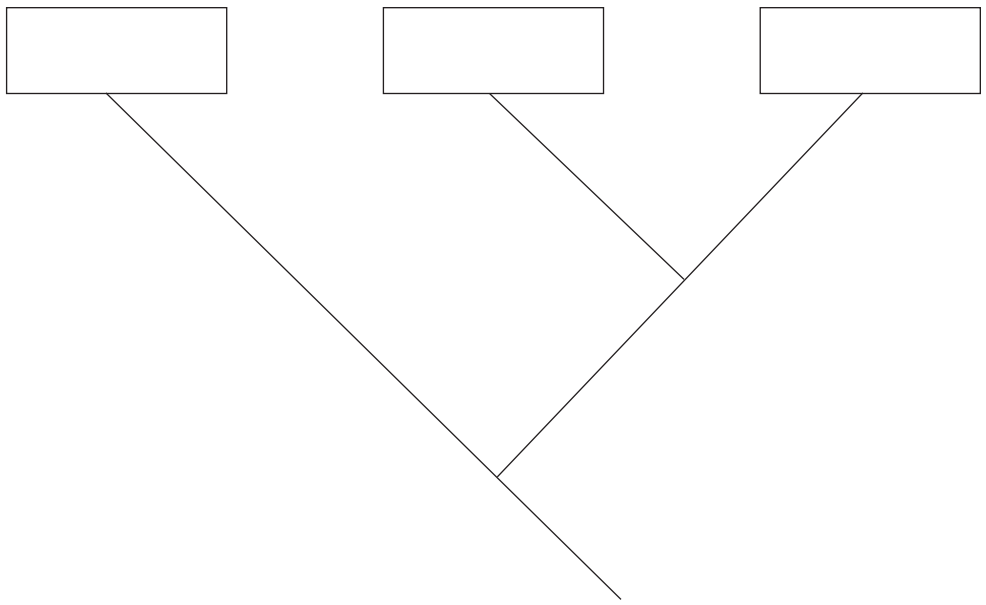


FIGURE 6: FISH WITH SCALES

Channel catfish
Ictalurus albidus

Scaleless

Bluefin tuna
Thunnus thynnus

Ctenoid scales
Have a toothed edge

Swordfish
Xiphias gladius

Ctenoid scales
Have a toothed edge

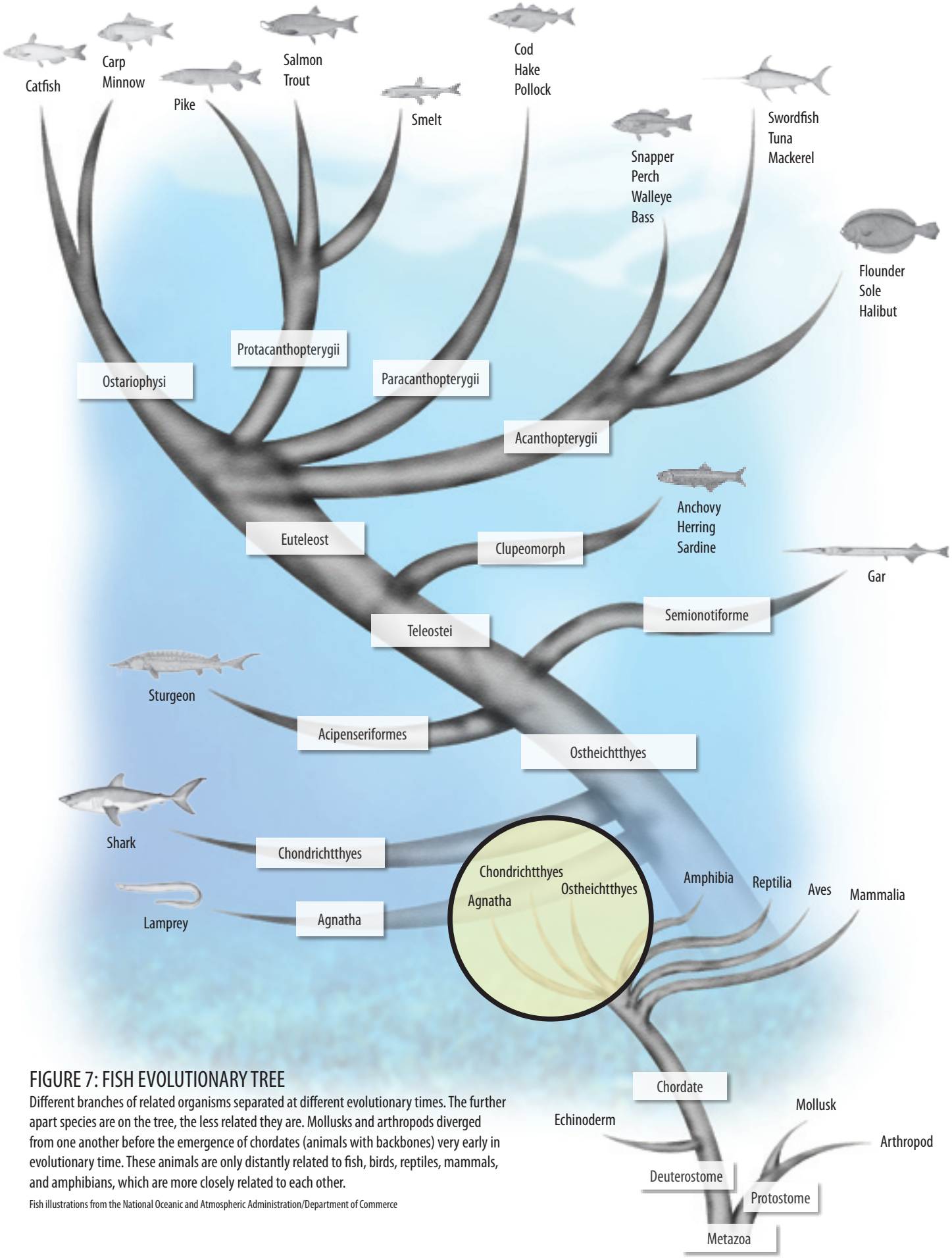


FIGURE 7: FISH EVOLUTIONARY TREE

Different branches of related organisms separated at different evolutionary times. The further apart species are on the tree, the less related they are. Mollusks and arthropods diverged from one another before the emergence of chordates (animals with backbones) very early in evolutionary time. These animals are only distantly related to fish, birds, reptiles, mammals, and amphibians, which are more closely related to each other.

Fish illustrations from the National Oceanic and Atmospheric Administration/Department of Commerce

BACKGROUND FOR THE WET-LAB

Proteins account for more than 50% of the dry weight of most cells, and they are instrumental in almost everything cells do. Proteins are used for structural support, storage, transport of substances, signaling from one part of the organism to another, and movement and defense against foreign substances. In addition, as enzymes, proteins selectively accelerate chemical reactions in cells.

KEY CONCEPT

Gel electrophoresis is a group of techniques used to separate molecules based on physical characteristics such as size, shape, or isoelectric point.

In gel electrophoresis, separation of charged molecules is achieved by subjecting the charged molecules to an electric current that forces them to migrate through a matrix (the gel). The two types of gel matrices used in molecular biology applications are agarose and polyacrylamide. Agarose is commonly used to separate large fragments of DNA. Polyacrylamide has a greater resolving power and is commonly used for separating proteins.

The behavior of molecules during gel electrophoresis depends on their size, shape, and net charge. Linear DNA molecules have uniformly negatively charged backbones and a shape that normally varies only in its length, so that migration is directly dependent on the size of the DNA fragment. With proteins, the story is different. The net charge of a protein is dependent on its amino acid content; proteins can carry a positive net charge, negative net charge, or they may be neutral. Similarly, the shapes of proteins vary widely. Furthermore, a protein may consist of several polypeptide sub-units held together by hydrogen bonds, hydrophobic interactions, and/or disulfide bridges. Therefore if proteins in their native configurations are electrophoresed, they will not all necessarily migrate in the same direction, and the distances migrated will not be solely a function of their sizes. Thus gel electrophoresis of native proteins cannot be used to determine molecular weights of proteins, but it can provide other information on characteristics of the protein in a mixture.

To make protein migration rates a function of molecular weight, it is necessary to impose a uniform shape and charge on all proteins in a mixture. This goal can be largely achieved by treating the protein mixture with the detergent sodium dodecyl sulfate (SDS). If a sample mixture is treated with hot SDS, this disrupts all the hydrogen bonds maintaining the protein's three-dimensional shape. If the sample is simultaneously treated with a reducing agent, such as beta-mercaptoethanol, disulfide bridges will also be broken, leaving the protein a linear chain of amino acids. The SDS binds to the protein backbone without regard to amino aid sequences, imparting a uniform negative charge to the molecules. Under these conditions, all the proteins in a mixture assume the same shape and charge. During electrophoresis, they migrate toward the positive pole at a rate proportional to the log 10 of their molecular weights.

Gel electrophoresis of proteins does not provide any direct information about amino acid sequences, and so cannot be used in a precise way to reconstruct evolutionary history. The overall protein fingerprints obtained from different species are more similar when closely related species are compared. The evolution of different groups of fish and the varying degrees to which they are related are a topic of ongoing study.

WET-LAB ENGAGEMENT

ENGAGE

To demonstrate the concept of protein electrophoresis, have three students come to the front of the room. Present each student with one of the three cards following this section (also on the module CD). Each card represents a protein molecule with a different molecular weight. Have students move as their proteins would move through the polyacrylamide gel based on their molecular weight. The smaller the mass of the protein molecule, the farther it moves towards the opposite end of the gel.

Three protein cards:

Protein	**Molecular weight, Dalton, D**
1. Myosin (H-chain)	200,000
2. Ovalbumin	43,000
3. β-lactoglobulin	18,400 (smaller mass moves farther toward the positive end)

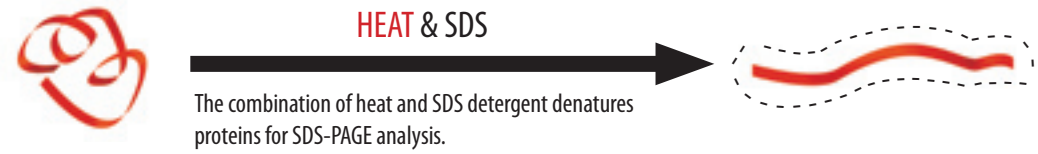
Three factors affect the movement of molecules through a gel during electrophoresis:

• The size of protein molecules: The smaller the protein, the farther down the polyacrylamide gel it will move. The size of a protein molecule is expressed by its molecular weight (in Daltons). Most proteins have masses on the order of thousands of daltons, so we measure them in kilodaltons (kD).

• Charge density of the protein: A ratio of a protein's electrical charge and mass. Charge density affects a protein's mobility through a gel during electrophoresis. Since each protein is made of a unique combination of amino acids, each of which may have a positive, negative, or neutral charge, the net charge of each protein is naturally different.

• Protein structure: Secondary, tertiary (which result from protein folding), and quaternary structure must be disrupted to separate proteins by size. The combination of heat and the detergent SDS denatures the protein's structure. The intrinsic charges of proteins are obscured by placing a strong anionic detergent SDA in both the sample buffer and the gel-running buffer. SDS binds to and coats the proteins and keeps them as denatured linear chains (see Figure 8). In this form, proteins migrate in polyacrylamide gels as if they have equivalent negative charge densities, and mass becomes the only variable affecting the migration rate of the protein. This technique is called SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

FIGURE 8: Sodium Dodecyl Sulfate



The combination of heat and SDS detergent denatures proteins for SDS-PAGE analysis.

Discussion Questions:

What is a protein?

Proteins are charged molecules; one of the complex organic chemical compounds that form the basis of living tissues; consist of long chains of amino acids connected by peptide bonds to form one or more polypeptides. Examples are enzymes, hemoglobin, and antibodies.

How do proteins function in organisms?

Proteins determine how an organism functions, what it eats, how it looks, and where it lives. Proteins form the basis of living tissues and carry out the thousands of chemical reactions necessary to maintain life.

Do humans and fish have the same proteins?

Actin and Myosin make up the structure and function of muscle found in all animals.

WET-LAB EXPLORATION

EXPLORE

(Depending on class time and ability, you may prepare samples in advance or guide students through the preparation.) Before entering the lab, review safety procedures. Give each student a copy of the lab procedure quick guide. Remind them to keep accurate records. In the lab, students will use the technique of protein electrophoresis to discover the relatedness of five types of fish. Using demonstration equipment, briefly describe the process used in the lab.

The purpose of the gel is to act as a medium to slow the rate of movement of the protein molecules, and to provide a lane for each sample to move in a straight line, much like a track. The sample loading buffer contains SDS and dithiothreitol (DTT), which will disrupt all the hydrogen bonds and disulfide bridges leaving the proteins linear with an overall uniform negative charge.

Add samples of fish proteins to designated wells.

Close the lid of the electrophoresis chamber and turn on the electricity.

After 30 minutes, remove the gel from the electrophoresis chamber and place it in a weigh boat for staining. (10 minutes)

Return the stain to the original container.

Flood the gel with destain solution and let it sit overnight until the bands are clearly visible.

WET-LAB EXPLANATION

EXPLAIN

After the electrophoresis process, students will analyze the gels. The protein profile for each fish will vary. To facilitate discussion, choose a representative gel. Place the results on an overhead projector. Highlight the bands with a marker. Some sample questions for discussion are as follows:

How would you identify the five proteins based on their movement through the gel?

Protein	Molecular weight, Dalton, D
1. Myosin (H-chain)	200,000 (largest mass travels least)
2. Bovine serum albumin	68,000
3. Ovalbumin	43,000
4. Carbonic anhydrase	29,000
5. β-lactoglobulin	18,400 (smallest mass travels farthest)

Which fish seem to have similar protein profiles? How many proteins do they have in common?

Which fish do not have similar protein profiles? What does this tell you about the relatedness of the fish?

WET-LAB EVALUATION

EVALUATE

Based upon their findings, ask students to place the names of the five fish on a hypothetical evolutionary tree. Lead a discussion with the class regarding their decisions for placement of fish on the hypothetical evolutionary tree. Remind students that gel electrophoresis of proteins does not provide any direct information about amino acid sequences, and so cannot be used in any precise way to reconstruct evolutionary history. The evolution of different groups of fish and the varying degrees to which they are related are topics of ongoing study.

FROM FINCHES TO FISHES WET-LAB: FISH PROTEIN FINGERPRINTING BY GEL ELECTROPHORESIS

PURPOSE

To compare proteins from five different fish to determine phylogenic relationships.

OBJECTIVES

- To perform gel electrophoresis to identify proteins present in each of five samples extracted from fish.
- To interpret the results of electrophoresis.
- To make inferences about the relatedness of fish based on the similarities of their proteins profiles.
- To demonstrate the process and concept of protein electrophoresis.

TEACHER PREPARATION

Obtain fish samples and distilled water—trip to grocery store.
Set up student and teacher workstations.

Student Workstations

1.5 ml micro test tube	6 each	☐
1.5 ml screw-cap micro test tube	7 each	☐
Micropipettes and tips or disposable pipettes	1 each	☐
Fish samples (5 types)	1 gram each	☐
Indelible marking pen, fine point	1	☐
Laemmli sample buffer	1.5 ml	☐
Knife or scissors to cut fish samples	1	☐

Teacher's (Common) Workstation

Water bath or hot plate set to 95° C	1	☐
Laemmli sample buffer — 30 ml	1	☐
Actin and myosin standards	1 vial	☐
Precision Plus Protein Kaleidoscope prestained standards — 500µl	1 vial	☐

ACTIN AND MYOSIN STANDARD

A control sample containing actin and myosin proteins is provided in a lyophilized form that is stable at room temperature. Store these proteins, along with the Precision Plus Protein Kaleidoscope standards, in the freezer for long-term safe-keeping. To rehydrate the sample, add 500 µl of Bio-Rad Laemmli sample buffer to the vial and incubate it at room temperature for five minutes. Transfer the rehydrated actin and myosin sample to a labeled screw cap tube using a disposable plastic transfer pipet. Like the fish protein samples, the actin and myosin samples must be heated for five minutes at 95°C before loading on gels.

LABORATORY DAY 1:
SAMPLE PREPARATION – MUSCLE PROTEIN EXTRACTION

PROCEDURE

1. Label (with indelible pen) 1.5 ml flip-top microtubes with the names of the fish samples to be analyzed. There should be one labeled tube for each fish sample being prepared for electrophoresis.

2. Add 250 µl of Laemmli sample buffer to each labeled tube.

3. For each sample, obtain a piece of fish muscle (avoid skin, fat, and bones) approximately 0.25 x 0.25 x 0.25 cm^3, and transfer it to the appropriately labeled microtube. Close the lid.

4. Gently flick the microtube 15 times with your finger to agitate the tissue in the sample buffer.

5. Incubate the samples for 5 minutes at room temperature to extract and solubilize the proteins.

6. Pour the buffer containing the extracted proteins, but not the solid fish piece, to a labeled 1.5 ml screwcap tube. Note: It's not necessary to transfer all of the fluid to the screwcap tube, since only a small volume (< 20 µl) is actually needed for gel loading.

7. Obtain aliquots of the Kaleidoscope (KS) and actin and myosin (AM) standards from your teacher.

8. Heat the fish samples and the actin and myosin (AM) sample in their screwcap tubes for 5 minutes at 95°C to denature the proteins in preparation for electrophoresis.

9. Store the samples at room temperature if they are to be loaded onto gels within 3-4 hours, or store them at -20°C for up to several weeks.

LABORATORY DAY 2: ELECTROPHORESIS

So far you have extracted, denatured, and given the proteins from fish muscle tissue a negative charge. Now they can be separated according to their molecular weights using gel electrophoresis.

Electrophoresis: gel loading, running, and staining

PURPOSE OF THIS LABORATORY

Generate profiles for various fish species via electrophoresis of extracted protein samples.
Procedure Overview:
1. Reheat fish and actin and myosin standard controls
2. Set up electrophoresis gel boxes
3. Load and run gels
4. Stain gels to visualize protein bands

Laboratory checklist

Student Workstations

Fish protein extracts prepared on laboratory day 1	5 each	☐
Prot/Elec pipet tips for gel loading	7 tips	☐
Mini-PROTEAN® 3 electrophoresis module (gel box — runs one or two gels)	1	☐
Buffer dam (if running only one gel/box)		☐
Power supply (200 V constant)	1	☐
2-20 µl micropipette	1	☐
Ready Gel precast gel, 15% – 10 wells	1	☐
Sample loading guides – for 10 well comb	1	☐
Thin metal weighing spatula	1	☐

Teacher's (Common) Workstation

**Actin and myosin standard sample, rehydrated	1 vial	☐
Precision Plus Protein Kaleidoscope prestained standards	1 vial	
1X Tris-glycine-SDS (TGS) electrophoresis buffer	As needed	☐
Bio-Safe Coomassie stain for proteins	As needed	☐
Staining trays		☐

**You may already have aliquots at student stations

LABORATORY DAY 2

STEP 1: PREPARE SAMPLES, ELECTROPHORESIS GELS, AND GEL BOXES

Note: Teachers may have already assembled the gel boxes. If not, follow these instructions.

1. Reheat frozen samples at 80-95°C for 2-5 minutes to redissolve any precipitated detergent.

2. Make sure the comb and the tape along the bottom of the Ready Gel cassette have been removed. If two gels are to be run in one electrophoresis box, place a Ready Gel cassette on each side of the electrode assembly, with the short plates facing the inside of the assembly. If you are running only one gel in the box, place a Ready Gel cassette on one side of the electrode assembly and a buffer dam on the other side. Be sure to place the side of the buffer dam that says "BUFFER DAM" toward the electrode assembly.

3. Open the gates (cams) on the front of the clamping frame. Hold the two Ready Gel cassettes, or one Ready Gel cassette and buffer dam, against the electrode assembly and slide the electrode assembly into the clamping frame.

4. Press down on the outer edge of the electrode assembly, not the gels, while closing the cams of the clamping frame to ensure a seal on the bottom edge of each cassette.

5. Place the assembled clamping frame containing the gel(s) into the gel box tank. Fill the upper buffer chamber, the space between the two gels, with ~150 ml 1X TGS electrophoresis buffer, so the buffer level is above the inner short plates. Check for leaks. If the assembly is leaking, remove the assembled clamping frame, pour off the buffer, reopen the cams, and push down on the electrode assembly again while closing the cams.

6. Pour ~200 ml of 1X TGS electrophoresis buffer into the lower buffer chamber, or tank. Double-check the buffer fill level within the upper buffer chamber.

Note: If leakage of the upper buffer cannot be corrected by reassembling the clamping frame in Step 4, the outer chamber can be filled to above the inner small plates, to equalize the buffer levels in both reservoirs. This requires approximately 900 ml of 1X TGS electrophoresis buffer.

STEP 2: LOAD AND RUN GELS

Place a yellow sample loading guide on the top of the electrode assembly. The guide will direct the pipet tip to the correct position for loading each sample in a well.

Assign samples to wells, loading samples in middle of the gel, where separation is best with the standards on each side. For example, for five fish samples on a 10 well gel, you may choose to follow this guide:

Lane	Volume	Sample
1	Empty	None
2	Empty	None
3	5 µl	Precision Plus Protein Kaleidoscope prestained standard
4	10 µl	Fish sample 1
5	10 µl	Fish sample 2
6	10 µl	Fish sample 3
7	10 µl	Fish sample 4
8	10 µl	Fish sample 5
9	10 µl	Actin and myosin standard
10	Empty	None

To load each sample, use a thin, gel loading micropipette tip to withdraw 10 µl of each protein sample from its tube and gently transfer it into the designated gel well. After loading all samples, remove the sample loading guide, place the lid on the tank, and insert the leads into the power supply, matching red to red and black to black. Set the voltage to 200V and run the gels for 30 minutes.

Record your samples here:

Lane Sample

1. Precision Plus Protein Kaleidoscope prestained standard

2. Precision Plus Protein Kaleidoscope prestained standard

3. Precision Plus Protein Kaleidoscope prestained standard

4. _____

5. _____

6. _____

7. _____

8. _____

9. Actin and myosin standard

10. Precision Plus Protein Kaleidoscope prestained standard

STEP 3: STAIN AND VISUALIZE THE PROTEINS

Gel staining

1. When gels are finished running, turn off the power supply and disconnect the leads. Remove the lid and lift out the electrode assembly and clamping frame.

2. Pour out the running buffer from the electrode assembly. Open the cams and remove the gel cassettes.

3. To keep the gel free of contamination from your fingertips, wear gloves to handle the gels from this point on. Lay a gel cassette flat on the bench with the short plate facing up. Cut the tape along the sides of the gel cassette.

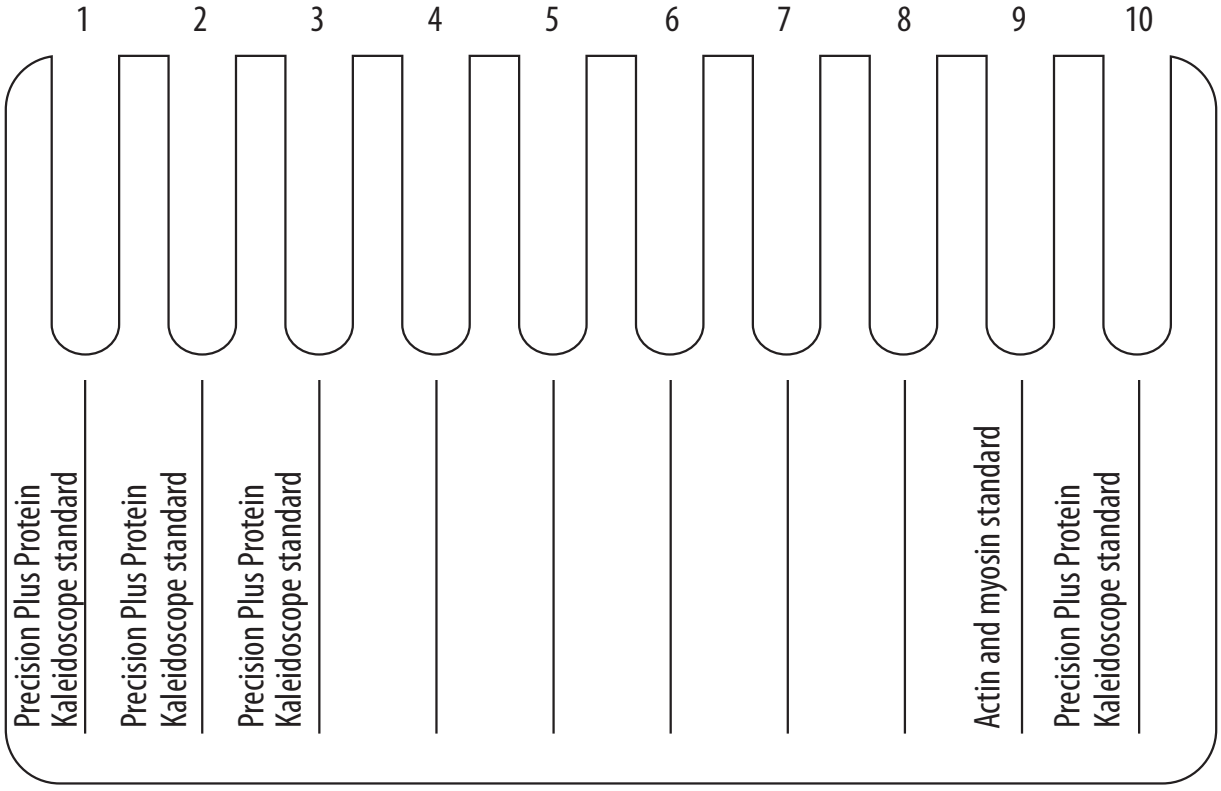
Carefully pry apart the gel plates, using a spatula or your fingertips. The gel will usually adhere to one of the plates. Transfer the plate with the gel adhering to it to a tray containing Bio-Safe Coomassie stain, allowing the liquid to detach the gel from the plate. The gel may also be lifted directly (and gently!) from the plate and placed into the stain.

4. Allow the gels to stain for one hour, with shaking if available.

5. Your teacher will discard the stain and replace it with a large volume of water to destain the gel overnight.

Name _____ Weigh Boat number _____

FROM FINCHES TO FISHES: DATA/OBSERVATION SHEET



FOCUS QUESTIONS

1. What are some important roles that proteins play in organisms?

2. Why did we apply heat and SDS buffer to the protein samples?

3. Why did we use a polyacrylamide gel instead of an agarose gel?

4. Which protein will travel further, a smaller protein or a larger protein?

5. What will the resulting protein bands help us to determine?

PROTEIN PROFILER MODULE — QUICK GUIDE

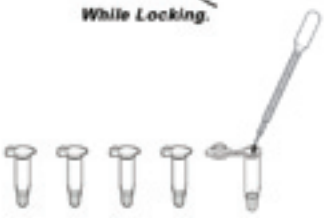
From Bio-Rad's Comparative Proteomics Kit I: Protein Profiler Module

1. Setup Mini-PROTEAN 3 gel box and add 1x TGS electrophoresis buffer to the chamber.

2. Label one 1.5 ml flip-top micro tube for each of five fish samples. Also label one screwcap microtube for each fish sample.

3. Add 250 µl of Bio-Rad Laemmli sample buffer to each labeled flip-top micro tube.

4. Cut a piece of each fish muscle about 0.25 x 0.25 x 0.25 cm^3 and transfer each piece into a labeled flip-top micro test tube. Close the lids.



5. Flick the microtubes 15 times to agitate the tissue in the sample buffer.

6. Incubate for five minutes at room temperature.

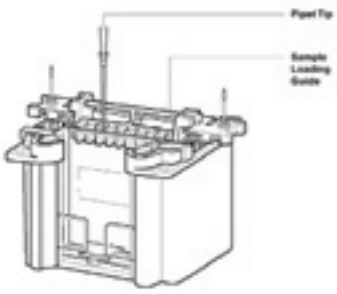
7. Carefully transfer the buffer by pouring from each flip-top microtube into a labeled screwcap microtube. Do not transfer the fish!

8. Obtain the Kaleidoscope prestained standards (KS) and the actin and myosin standard from your teacher.

9. Heat the fish samples and the actin and myosin standard (AM) in screwcap microtubes for five minutes at 95°C.

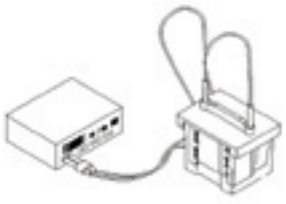
10.Load your gel:

Lane	Volume*	Sample
1 & 2	empty	empty
3	5 µl	Precision Plus Protein Kaleidoscope standards (KS)
4	10 µl	fish sample 1
5	10 µl	fish sample 2
6	10 µl	fish sample 3
7	10 µl	fish sample 4
8	10 µl	fish sample 5
9	10 µl	Actin and myosin standard (AM)
10	empty	empty



* — DESTINY has found that doubling the volume to 20 µl helps students get enough protein out of the pipettes.

11. Electrophorese for 30 minutes at 200 V in 1x TGS electrophoresis buffer.



12. After electrophoresis, remove gel from cassette and transfer gel to a container with 25 ml Bio-Safe Coomassie stain per gel and stain gel for 1 hour, with gentle shaking for best results.

13. Discard stain and destain gels in a large volume of water for at least 30 minutes to overnight, changing the water at least once. Blue-stained bands will be visible on a clear gel after destaining.

14. Dry gels using GelAir™ cellophane.

ANALYZING RESULTS OF GEL ELECTROPHORESIS AND DETERMINING SIGNIFICANCE OF DATA

MATERIALS NEEDED

- 1 Protein Fingerprinting: Analyzing Results of Gel Electrophoresis handout for each student
- 1 What Do the Bands Reveal? What Is the Significance of Your Data? handout for each student
- Students' gels from wet-lab
- 1 ruler per student
- 1 magnifying glass per student

BACKGROUND INFORMATION FOR THE TEACHER

Similarities and differences between protein fingerprints are easily spotted. By comparing the banding pattern of the shark with other fish it is clear that this fish is dissimilar from all of the others. This is consistent with the proposed fish evolutionary tree, since the shark belongs to the Class Chrondrichthyes — cartilaginous fishes — as compared to the other species, which belong to the Class Osteichthyses — bony fishes. Salmon and trout, which are on the same branch, have many similar bands. Swordfish and tuna, located on the same branch, show some similarities in their protein bands. By contrast, salmon and catfish, located on different branches, reveal significant differences in their banding patterns. The actin and myosin standard is included as a reference to help identify the major, conserved muscle proteins and to serve as a positive control for gel analysis. This protein consists of myofibrils isolated from rabbit skeletal muscle. The Precision Plus Protein Kaleidoscope prestained standards are included to provide a means of practice for loading the samples into the polyacrylamide ready gels and to help create a standard curve when graphing the molecular weights of proteins.

INSTRUCTIONS FOR TEACHERS

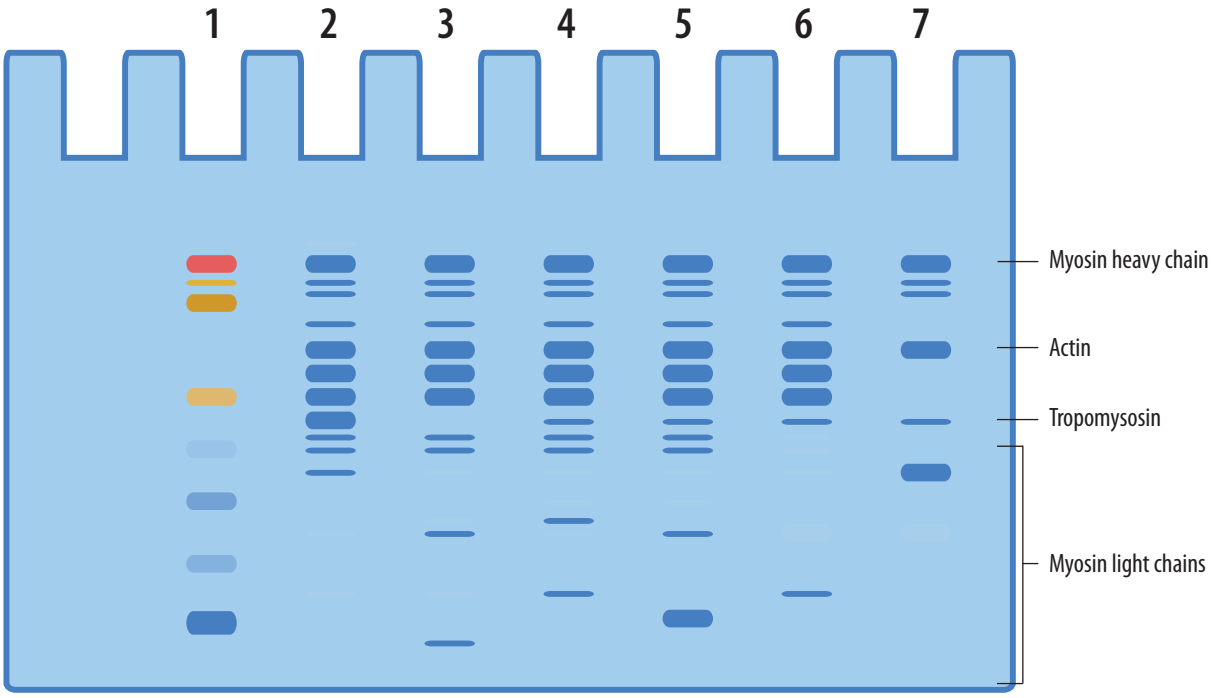
Distribute the two handouts to the class (copy from notebook or print from module CD). Use the Protein Fingerprinting handout to review the process for analyzing results of gel electrophoresis. Because the diagram on this handout is clear and easy to read, it provides a good opportunity to practice the technique of counting bands.

After practicing, students should count the bands on their own gels, filling out the chart on the What Do the Bands Reveal? handout. After they have completed the chart and read the additional information on the significance of the data, have them answer the three questions and then review the results together as a class.

PROTEIN FINGERPRINTING: ANALYZING RESULTS OF GEL ELECTROPHORESIS

Evolutionary biologists make hypotheses about relationships among different groups of organisms based on how similar they are, in terms of both morphological and molecular traits. Below are the results of a gel electrophoresis procedure, used to create protein "fingerprints" of several species of fish. By comparing the number of protein bands that each kind of fish has in common with one another, you can make your own hypothesis about which of these fish are most closely related.

SAMPLE PROTEIN FINGERPRINTS



Lane: 1. Precision Plus Protein Kaleidoscope standards
2. Shark
3. Salmon
4. Trout
5. Catfish
6. Swordfish
7. Actin and myosin standard

WHAT DO THE BANDS REVEAL?

Using a ruler or straight edge, carefully count the number of bands that each kind of fish has in common with the others. Do not include any of the bands in the actin and myosin standards in your counts, since every kind of fish has these bands. Then fill out the table below with your results. Which of these fish do you think are most closely related?

COMPARISON OF NUMBER OF SIMILAR PROTEIN BANDS FOUND IN FISH

	Shark	Salmon	Trout	Catfish	Swordfish
Shark					
Salmon					
Trout					
Catfish					
Swordfish					

WHAT IS THE SIGNIFICANCE OF YOUR DATA?

To make meaningful inferences about evolutionary relationships among the sample fish species, use a ruler and a magnifying glass to compare the banding patterns.

The actin and myosin standard is included as a reference to help identify the major, conserved muscle proteins and to serve as a positive control for gel analysis. The Precision Plus Protein Kaleidoscope prestained standards are included to provide a means of practice for loading the samples into the polyacrylamide ready gels and to help create a standard curve when graphing the molecular weights of protein.

Gel electrophoresis is a powerful tool for separating and visualizing the individual proteins in complex samples like muscle tissue. The two types of molecules most often analyzed by electrophoresis are nucleic acids, like DNA, and proteins. Electrophoresis not only lets you determine how many distinct types of molecules are in the sample, it can also tell you their sizes, which can be a clue to its identity.

The procedure that you have completed displays a profile of protein composition in the muscle tissue of different fish. Since proteins are a reflection of an organism's DNA, variations in these composition profiles indicates variations in DNA sequences. Evolutionary relationships among species are inferred from the degree of genetic (DNA) similarity among them. The protein fingerprints you've generated, indirectly representing the genetic compositions of your chosen fish species, are molecular-level indicators of evolutionary relationships.

Similarities and differences between protein fingerprints are easily spotted.

1. Compare the banding pattern of the shark with other fish samples. How might you explain any differences?

2. How does the salmon and catfish compare? Are they located on the same branch?

3. Do the swordfish and tuna, located on the same branch, show similarities in their protein bands?

INTERPRETING THE BANDS COLLECTIVELY: QUALITATIVE COMPARISONS OF PROTEIN PROFILES

From Bio-Rad's Comparative Proteomics Kit I: Protein Profiler Module

1. Is there any variation among the protein profiles of your samples?

2. How would you distinguish the protein profiles of different species from each other?

3. What are possible explanations for this variation?

4. Which samples are most alike?

Tape your gel or a photocopy of it in the box. The bands are the data upon which your analysis will be based.

ANALYSIS AND INTERPRETATION OF RESULTS: DETAILED GEL ANALYSIS

From Bio-Rad's Comparative Proteomics Kit I: Protein Profiler Module

Does your molecular evidence support or refute your predictions?

Each protein band that a fish has in common with another fish is considered a shared characteristic. A fish family tree, or cladogram, can be constructed based on proteins bands that the fish have in common. Cladistic analysis assumes that when two organisms share a common characteristic that they also share a common ancestor with that same characteristic.

Create a cladogram using your results to find out.

From your gel you can create a cladogram based on proteins that the fish have in common. You can then determine whether your cladogram supports your predictions and/or matches the evolutionary relatedness of the fish species determined by morphological analysis in the evolutionary tree provided. Each protein band that a fish has in common with another fish is a shared characteristic. Cladistic analysis assumes that when two organisms share a characteristic, they had a common ancestor that had that characteristic, and this can be represented as a node on a cladogram with two branches coming from that node representing the descendent organisms.

In this exercise you will define the shared characteristics (i.e., make a list of all the different proteins in fish muscle), find which proteins (characteristics) are shared between fish, and construct a cladogram based on your data.

PROCEDURES

Generate a standard curve to calculate protein molecular weights

(Optional) Although it is not strictly necessary for this exercise, you may want to create a standard curve from your gel and determine the actual size of each protein band.

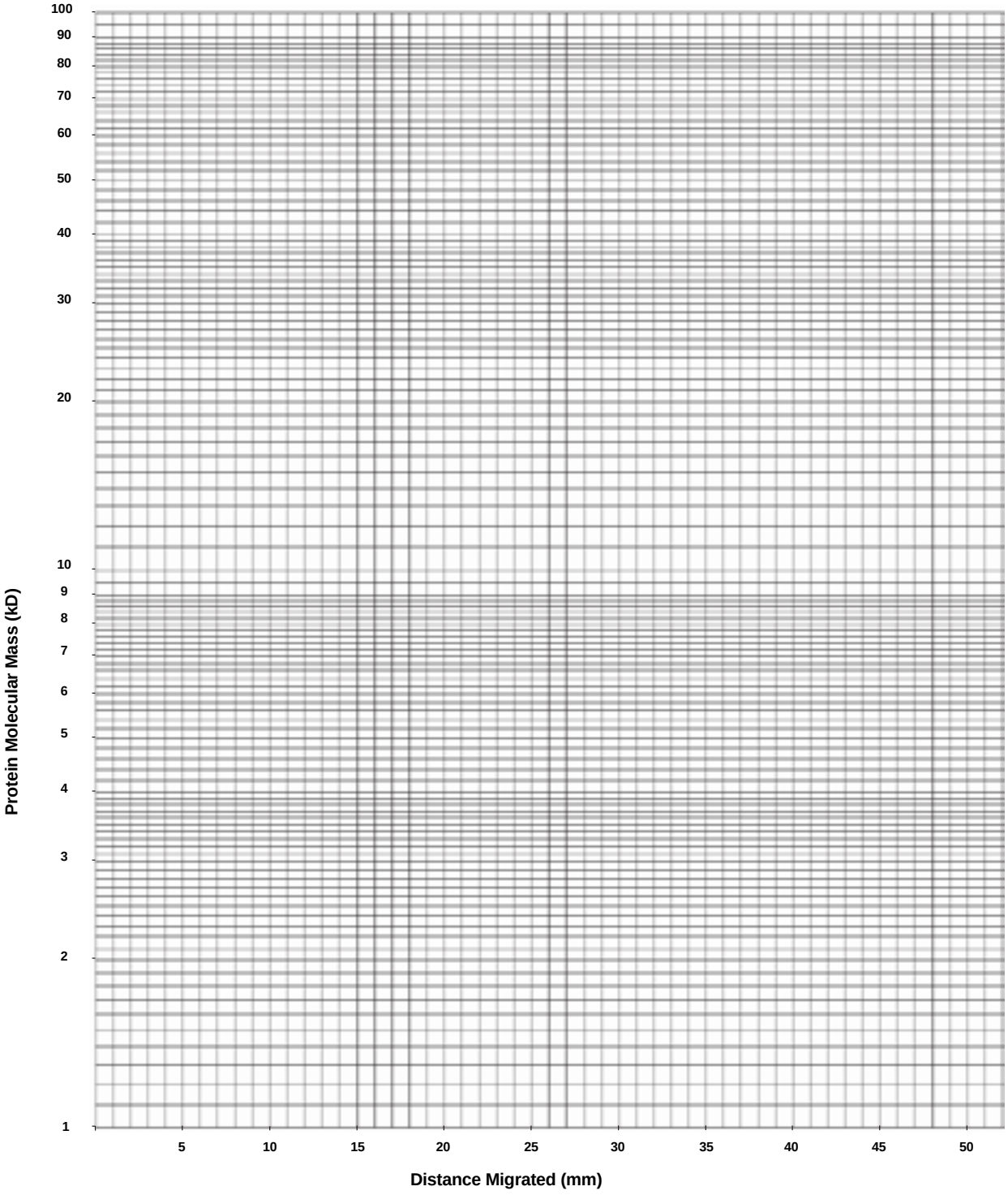
Alternatively, the cladogram can be generated just using the distance in millimeters the different protein bands have migrated from the wells of the gel.

To create the standard curve measure and record the distances the five visible protein bands contained in the Precision Plus Protein Kaleidoscope prestained standards. Start from the green 37 kD band down to the yellow 10 kD band that has migrated from the wells. Accuracy to 0.5 mm is required.

Precision Plus Protein Kaleidoscope Prestained Standards Molecular Weight (kD)	Distance Migrated (mm)
37	
25	
20	
15	
10	

On the graph paper provided, plot the distances migrated in mm on the x-axis against the molecular weight of the bands in kD on the y-axis as a scatter plot.

Draw a line through the points. On a logarithmic scale, plotting the molecular weights against the distances migrated for each protein in the standard should result in a linear (straight line) curve. Alternatively, you can use a graphing computer program to generate the chart and make a line of best fit (or a trend-line) through these points and to formulate an equation to calculate the MW of the unknown proteins on the gel.



Define the characteristics (proteins) of the different fish

Make a horizontal line on the dried gel (or gel image) between the 37 kD (green) and 25 kD (pink) markers below the fat bands that occur at around 30 kD (see gel above). Then, for each band below the line for each fish sample, measure the distance the protein band has migrated from the wells (and, if required, determine its size in kD using the standard curve or the formula generated from the standard curve) and record this data (see example below):

	Species A	Species B	Species C	Species D	Species E
Distance protein bands migrated (mm)	25, 26.5, 29, 36, 36.5, 39, 44, 52	26, 27.5, 29, 32, 34.5, 36.5, 37.5, 40.5, 42, 45	26, 27.5, 29, 29.5, 32, 34.5, 36.5, 37.5, 40.5, 42, 45, 46.5, 51.5	26, 27.5, 29, 32, 36.5, 38, 38.5, 41, 46, 47.5, 44, 47	26, 27.5, 30, 30.5, 33, 35.5, 37, 39, 39.5, 42

Record your data in the table below:

Distance protein bands migrated (mm)					

Determine which fish have each characteristic (protein)

In the blank table provided on page 108, use one row for every band size you have recorded and one column for each type of fish on your gel. Then make a mark in each cell of the table where the fish has that size band (see example on next page).

Example:

CHARACTERISTIC

Distance Migrated (mm)	Protein Molecular Mass (kDa)	Species A	Species B	Species C	Species D	Species E
25	32.5	X				
26	31.5		X	X	X	X
26.5	31.0	X				
27.5	30.0		X	X	X	X
28.5	29.1					
29	28.6	X	X	X	X	
30	27.6			X		X
30.5	27.1					X
32	25.6		X	X	X	
33	24.7					X
34.5	23.2		X	X		
35.5	22.2					X
36	21.7	X				
36.5	21.2	X	X	X	X	
37	20.7					X
37.5	20.2		X	X		
38	19.7				X	
38.5	19.3				X	
39	18.8	X				X
39.5	18.3					X
40.5	17.3		X	X		
41	16.8				X	
41.5	16.3					
42	15.8		X	X		X
43	14.8					
44	13.9	X				X
45	12.9		X	X		
46	11.9				X	
46.5	11.4			X		
47	10.9					X
47.5	10.4				X	
51.5	6.5			X		
52	6.0	X				
	COUNT	**8**	**10**	**13**	**10**	**12**

Record your data in the table below:

CHARACTERISTIC

Distance Migrated (mm)	Protein Molecular Mass (kDa)					

Find the number of characteristics shared by each of the fish

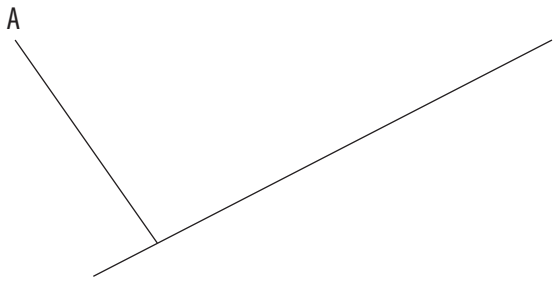
In the table below both the row and column headings are the types of fish. From the table on page 105, separately compare the number of bands (X's) in common with every other fish sample from your gel and put those numbers into the table below, such that each fish is individually compared with every other fish. In this example, species A and B have just two bands in common while species B and C have 10 bands in common. The table below will be the basis for drawing your cladogram.

	Species A	Species B	Species C	Species D	Species E
Species A	8	2	2	2	2
Species B		10	10	5	3
Species C			13	5	4
Species D				10	2
Species E					12

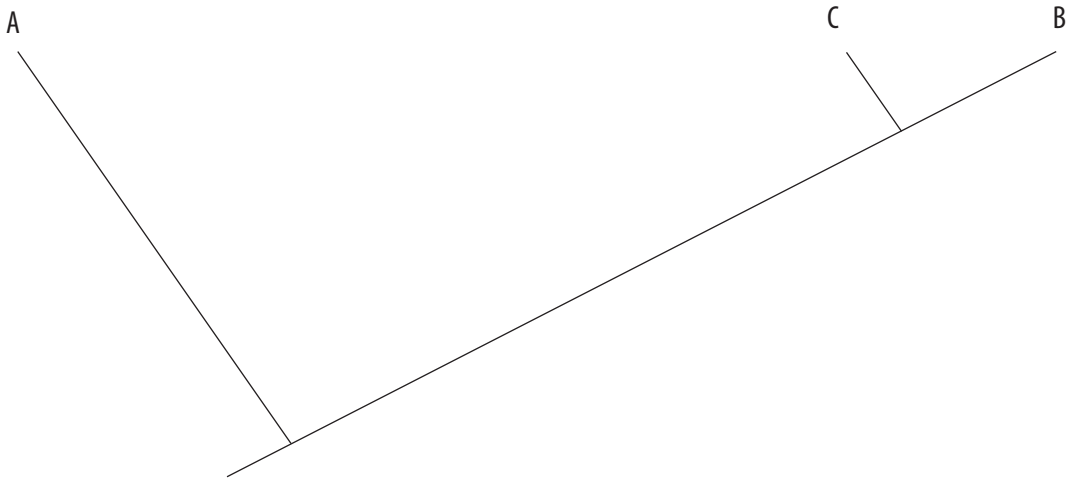
Record your data in the table below:

Construct your cladogram

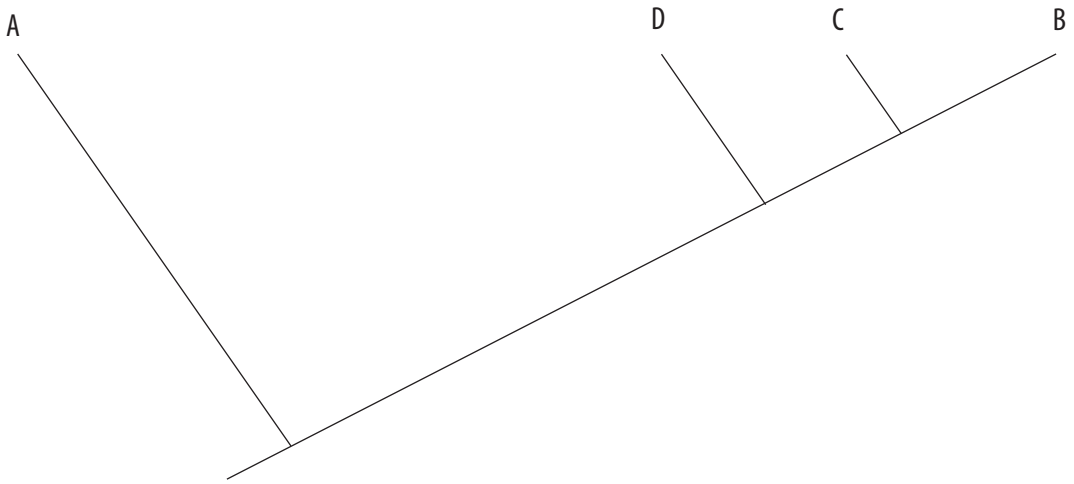
Now you are ready to construct your cladogram. First draw a line to form the trunk of your cladogram. Find the fish with the least bands in common. In the example above it is species A, which has only 2 bands in common with any of the other fish. Then draw a side branch off the line near the bottom of the trunk and label that branch with the fish's name, in this case, species A. This fish is the outlier, i.e., it is the least similar to any of the others. The node (where the side branch meets the trunk) represents an ancestor that is common to all the fish in this analysis.



Find the two fish with the most bands in common (in this example it is species B and C, which have 10 bands in common). Draw a side branch off the trunk near the top and label the two ends with the fishes' names, in this case, species B and species C (it doesn't matter which branch gets which label). The node represents a common ancestor of species B and species C that had all the same characteristics (proteins).

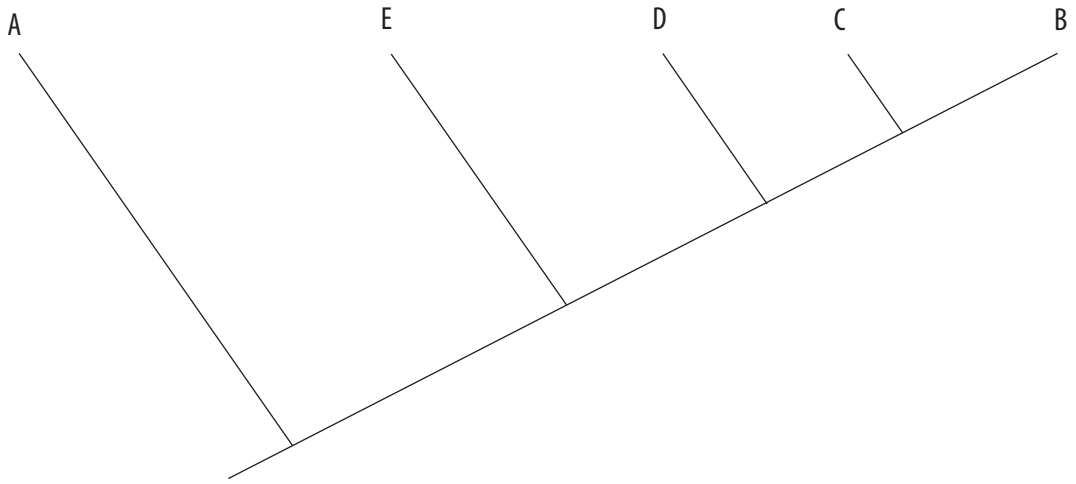


Identify those fish species with the next most bands in common. In this example, species D has five bands in common with species B and species C, which indicates species D is the same cladistic distance from B and C (i.e. species D is not more closely related to either B or C). Draw a branch further down the trunk. This node represents an ancestor that is common to species B, C, and D that had these 5 characteristic proteins.



The last fish to add to the cladogram in this example is species E, which shares four bands with species C, three bands with species B, and only two bands with species A and D. This fish may seem trickier to place than the others because it shares more characteristics with species B and C than it does with D, but D shares more characteristics with B and C than E does. So, to place this fish you might ask: Does species E share the five proteins that the common ancestor of species B, C, and D had? Answer (no).

Does species E share more proteins with B, C, and D than A? Answer (yes). Therefore, species E gets its own branch in between the D and A branches to indicate that it has more shared characteristics with B, C, and D than A, but fewer shared characteristics with B and C than D.



Using your own data, draw a cladogram below.

Compare your cladogram with your original predictions. Write your deductions below.

DISCUSSION QUESTIONS: ISN'T EVOLUTION JUST A THEORY?

From *PBS Evolution Series: Learning and Teaching Evolution*

1. How does the scientific meaning of a term like "theory" differ from the way it is used in everyday life?

2. Can the "facts" of science change over time? If so, how?

USING DATABASES TO OBTAIN REAL AMINO ACID SEQUENCE DATA TO CREATE CLADOGRAMS

From Bio-Rad's Comparative Proteomics Kit I: Protein Profiler Module

In order to determine how closely related species are, scientists often will study amino acid sequences of essential proteins. Any difference in the amino acid sequence is noted and a phylogenetic tree is constructed based on the number of differences. More closely related species have fewer differences (i.e., they have more amino acid sequence in common) than more distantly related species.

There are many tools scientists can use to compare amino acid sequences of muscle protein. One such tool is the National Center for Biotechnology Information protein databases (http://www.ncbi.nlm.nih.gov/). By entering the amino acid sequence of a protein you are interested in, the BLAST search tool compares that sequence to all others in its database. The data generated provides enough information to construct cladograms.

The purpose of this activity is to use data obtained from NCBI to construct an evolutionary tree based on the amino acid sequences of the myosin heavy chain. In this example we have input a 60 amino acid sequence from myosin heavy chain of rainbow trout and then pulled out matching sequences using BLAST, which include chum salmon, zebra fish, common carp, and bluefin tuna, and then compared each of these sequences with each other.

You may either use the data provided below or have your class go online and obtain their data directly by performing BLAST searches. A quick guide to performing BLAST searches is given at the end of this activity.

The data below was obtained by entering a 60 amino acid sequence from the heavy myosin chain of rainbow trout. The database search tool returned all sequences that were a close match. The results are formatted as such:

gi|755771|emb|CAA88724.1 myosin heavy chain [Oncorhynchus mykiss]
Length=698

Score = 119 bits (299), Expect = 2e-26
Identities = 60/60 (100%), Positives = 60/60 (100%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL

Sbjct 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60

The value for "identities" is the number of amino acids exactly in common, the value for "positives" is the number of amino acids that are similar to each other (such as serine and threonine), and the value for 'gaps' is the number of amino acid positions that are absent one of the sequences. "Query" is the original trout sequence, "Sbjct" is the aligned sequence, and the middle sequence shows the mismatches: a "+" indicates a positive and a space indicates a mismatch that is not a positive. There are resources on the NCBI website to help you understand more about the information a BLAST search generates.

The data on the following pages compares rainbow trout to salmon, zebra fish, carp, and tuna, and then compares salmon to zebra fish, carp, and tuna, then zebra fish to carp and tuna, and finally carp to tuna.

Use the data provided to determine how many amino acid differences exist between the organisms. Organize your data in charts.

Rainbow trout compared to chum salmon

gi|21623523|dbj|BAC00871.1| myosin heavy chain [Oncorhynchus keta]
Length=1937

Score = 119 bits (299), Expect = 2e-26
Identities = 60/60 (100%), Positives = 60/60 (100%), Gaps = 0/60 (0%)

Query 1
AKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL

Sbjct 1240
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 1299

Rainbow trout compared to zebra fish

gi|68360600|ref|XP_708916.1| PREDICTED: myosin, heavy polypeptide 1, skeletal muscle [Danio rerio]
Length=2505

Score = 108 bits (269), Expect = 6e-23
Identities = 52/60 (86%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAK NLEKMCRTLEDQLSE+K+KNDEN+RQ+ND+S QRARL TENGEFGRQLEEKEAL

Sbjct 1240
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL 1299

Rainbow trout compared to common carp

gi|806515|dbj|BAA09069.1| myosin heavy chain [Cyprinus carpio]
Length=955

Score = 104 bits (259), Expect = 8e-22
Identities = 51/60 (85%), Positives = 56/60 (93%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAK NLEKMCRTLEDQLSE+KTK+DENVRQ+ND++ QRARL TENGEF RQLEEKEAL

Sbjct 259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL 318

Rainbow trout compared to bluefin tuna

gi|1339977|dbj|BAA12730.1| skeletal myosin heavy chain [Thunnus thynnus]
Length=786

Score = 104 bits (259), Expect = 8e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAK+KGNLEKMCRT+EDQLSELK KNDE+VRQ+ND++GQRARL TENGEF RQ+EEK+AL

Sbjct 88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL 147

Chum salmon compared to common carp

gi|806515|dbj|BAA09069.1| myosin heavy chain [Cyprinus carpio]
Length=955

Score = 104 bits (259), Expect = 8e-22
Identities = 51/60 (85%), Positives = 56/60 (93%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAK NLEKMCRTLEDQLSE+KTK+DENVRQ+ND++ QRARL TENGEF RQLEEKEAL

Sbjct 259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL 318

Chum salmon compared to zebra fish

gi|68360600|ref|XP_708916.1| PREDICTED: myosin, heavy polypeptide 1, skeletal muscle [Danio rerio]
Length=2505

Score = 108 bits (269), Expect = 6e-23
Identities = 52/60 (86%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAKAK NLEKMCRTLEDQLSE+K+KNDEN+RQ+ND+S QRARL TENGEFGRQLEEKEAL

Sbjct 1240
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL 1299

Chum salmon compared to bluefin tuna

gi|1339977|dbj|BAA12730.1| skeletal myosin heavy chain [Thunnus thynnus]
Length=786

Score = 104 bits (259), Expect = 8e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKGNLEKMCRTLEDQLSELKTKNDENVRQVNDISGQRARLLTENGEFGRQLEEKEAL 60
VAK+KGNLEKMCRT+EDQLSELK KNDE+VRQ+ND++GQRARL TENGEF RQ+EEK+AL

Sbjct 88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL 147

Zebra fish compared to common carp

gi|806515|dbj|BAA09069.1| myosin heavy chain [Cyprinus carpio]
Length=955

Score = 108 bits (271), Expect = 4e-23
Identities = 53/60 (88%), Positives = 59/60 (98%), Gaps = 0/60 (0%)

Query 1
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL 60
VAKAKANLEKMCRTLEDQLSEIK+K+DEN+RQ+ND++AQRARLQTENGEF RQLEEKEAL

Sbjct 259
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL 318

Zebra fish compared to bluefin tuna

gi|1339977|dbj|BAA12730.1| skeletal myosin heavy chain [Thunnus thynnus]
Length=786

Score = 102 bits (253), Expect = 4e-21
Identities = 47/60 (78%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKANLEKMCRTLEDQLSEIKSKNDENLRQINDLSAQRARLQTENGEFGRQLEEKEAL 60
VAK+K NLEKMCRT+EDQLSE+K+KNDE++RQ+NDL+ QRARLQTENGEF RQ+EEK+AL
Sbjct 88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL 147

Common carp compared to bluefin tuna

gi|1339977|dbj|BAA12730.1| skeletal myosin heavy chain [Thunnus thynnus]
Length=786

Score = 104 bits (259), Expect = 9e-22
Identities = 49/60 (81%), Positives = 57/60 (95%), Gaps = 0/60 (0%)

Query 1
VAKAKANLEKMCRTLEDQLSEIKTKSDENVRQLNDMNAQRARLQTENGEFSRQLEEKEAL 60
VAK+K NLEKMCRT+EDQLSE+K K+DE+VRQLND+N QRARLQTENGEFSRQ+EEK+AL
Sbjct 88
VAKSKGNLEKMCRTIEDQLSELKAKNDEHVRQLNDLNGQRARLQTENGEFSRQIEEKDAL 147

Construct a table of your data containing the number of amino acid differences between each of the different fish.

	Rainbow trout	Chum salmon	Zebra fish	Common carp	Bluefin tuna
Rainbow trout					
Chum salmon					
Zebra fish					
Common carp					
Bluefin tuna					

Which two fish share the most amino acids in their myosin heavy chains based on your data?

Which two fish share the fewest amino acids?

Are there any fish that share more amino acids with each other than each does with the two fish in question one? If yes, which fish?

Construct a cladogram based on this data:

The myosin heavy chain of white croaker (Pennahia argentata) (BAB12571) has the following amino acid differences with the five fish above.

	Rainbow trout	Chum salmon	Zebra fish	Common carp	Bluefin tuna
White croaker	4	4	11	9	11

Add this fish to your cladogram and explain why you placed it where you did.

Taxonomic data can be derived from many sources: DNA sequences, protein sequences, morphology, and paleontology. Classification of organisms derives from these sources. Inconsistencies in the phylogenetic trees generated between molecular and taxonomic data emphasize why data from different sources is required to generate phylogenetic trees and why there is still much dispute in the field of phylogenetics on the correct placement of organisms within phylogenetic trees. The amount of work required to process the small amount of data provided here also emphasizes the need for skilled bioinformaticists to process and analyze the vast amount of data generated by genomic and proteomic research.

Examine the taxonomic classification of the fishes below and construct a phylogenetic tree based on that data. The large phylogenetic tree figure will be useful for this exercise.

Rainbow Trout (*Oncorhynchus mykiss*) Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.

Chum Salmon (*Oncorhynchus keta*) Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Protacanthopterygii; Salmoniformes; Salmonidae; Oncorhynchus.

Zebra Fish (*Danio rerio*) Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Danio.

Carp (*Cyprinus carpio*) Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Cyprinus.

Bluefin Tuna (*Thunnus thynnus*) Vertebrata; Euteleostomi; ctinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; Acanthomorpha; Acanthopterygii; Percomorpha; Perciformes; Scombroidei; Scombridae; Thunnus.

White Croaker (*Pennahia argentata*) Vertebrata; Euteleostomi; ctinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; canthomorpha; Acanthopterygii; Percomorpha; Perciformes; Percoidei; Sciaenidae; Pennahia.

Does the taxonomic classification support the molecular data?

Why do scientists need to examine multiple data sets before determining evolutionary relatedness?

QUICK GUIDE TO BLAST SEARCHING

Please note, this is a quick guide to obtain a list of fish myosin sequences, there are many refinements you can make to your search and many different ways to use BLAST searches.

Further information can be found on the NCBI website.
1) Go to http://www.ncbi.nlm.nih.gov/ and choose BLAST.

2) Choose Protein-Protein BLAST.

3) Enter your myosin sequence into the search box. Rainbow Trout Myosin Heavy Chain Protein Sequence (CAA88724):

VAKAKGNLEKMCRTLEDQLSELKTKNDEN-
VRQVNDISGQRARLLTENGEFGRQLEEKEAL

4) Leave the other fields as found and hit the BLAST button.

5) A new window should pop up. Hit the Format button.

6) After a short wait the BLAST results window will come up and may well be hundreds of pages long — don't worry. There should be a long list of sequences that produced significant alignments. Although the search may pick up hundreds of sequences, they are in order of homology, so the ones you are interested in should be in the first 25 or so.

7) Further down the BLAST results page, after the list of sequences, each sequence will be aligned with the original trout sequence (as shown in the example) so that you can see how the two compare.

8) To compare your second fish, say bluefin tuna, with the other fish, you must perform a second BLAST search with the tuna sequence to obtain the protein alignments of tuna with the other fish. Alternatively, you can align 5 protein sequences yourself from your original search in a word processing document (use Courier font, this aligns sequences because all the letters are the same width) and have your students manually compare them.

BLAST-SEARCHING QUESTIONS

Construct a simple phylogenetic tree based on the taxonomic data.

Does the taxonomic data support the molecular data? Please explain your answer.

Why do scientists need to examine multiple data sets before determining evolutionary relatedness?