

Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels

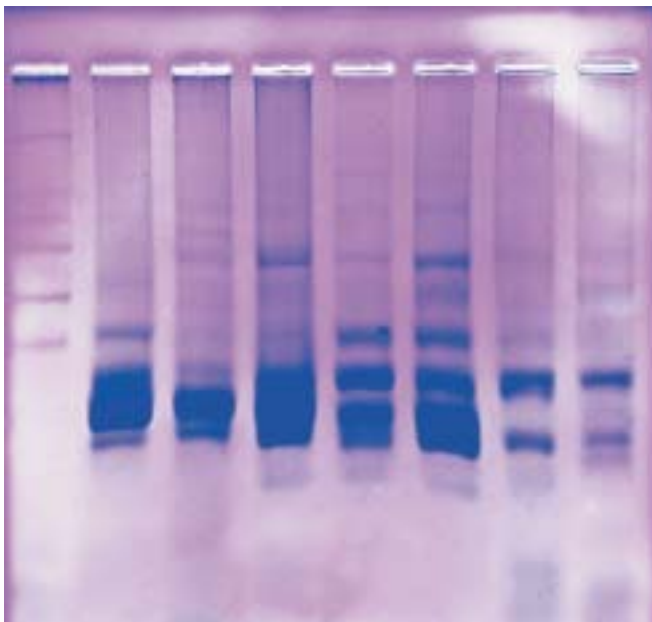
21-1255

Fish Protein Fingerprinting
on Agarose Gels Kit

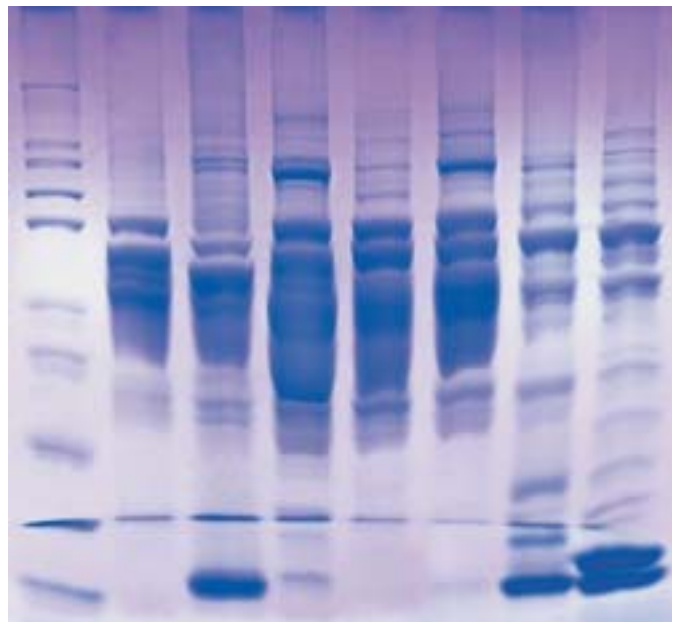
21-1260

Fish Protein Fingerprinting
on Polyacrylamide Gels Kit

TEACHER'S MANUAL WITH STUDENT GUIDE



Agarose gel



Polyacrylamide gel

Note: Upon receipt, store the fish protein extracts in a freezer (approximately -20°C). Store the protein size standard and pre-cast polyacrylamide gels in a refrigerator (approximately 4°C). All other materials may be stored at room temperature (approximately 25°C). Avoid repeated freezing and thawing of fish protein extracts. Keep fish protein extracts on ice while in use.

Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels

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Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels

Overview

This Teacher's Manual can be used with the *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255) as well as the *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260). Using these kits, students perform gel electrophoresis on extracted muscle protein mixtures from seven different species of fish. Electrophoresis of the protein extracts creates a unique pattern of bands for each fish species, called a protein fingerprint. Students compare the protein fingerprints from the seven different types of fish and, on the basis of their findings, hypothesize on the degree of relatedness of the fish. An Evolutionary Tree of Fish is provided to help analyze and interpret student results. It is assumed that students have prior knowledge of protein composition and DNA heritability.

The materials provided in the *Fish Protein Fingerprinting on Agarose Gels* kit are sufficient for running six agarose gels (6 stations). The materials provided in the *Fish Protein Fingerprinting on Polyacrylamide Gels* kit are sufficient for running four polyacrylamide gels (4 stations). However, the quantities of fish protein extracts, Tris-glycine-SDS buffer, and stain and destain solutions are sufficient for 6 gels. Additional gels, protein markers, staining trays, and gloves can be purchased separately.

Instruction Note: Two different electrophoresis and staining procedures are included in the Student Guide—one for use with agarose gels and one for use with polyacrylamide gels. The introduction and questions are the same regardless of which gels are used.

Objectives

- Students will learn laboratory techniques associated with protein gel electrophoresis.
- Students will learn how proteins can be used to study evolution.
- Students will hypothesize on the relatedness of different fish species by comparing fish protein fingerprints.
- Students will test their hypothesis by comparing their conclusions with those presented in the Evolutionary Tree of Fish provided.

Background

Proteins and Evolutionary Biology

Evolutionary biologists are interested in how individual species arose from earlier forms. A classic way of addressing this question is to compare the morphology of different living organisms to one another and to fossilized species. With the onset and advancement of molecular biology, it is now possible for evolutionary biologists to compare not only the physical form and structure of different organisms, but their DNA and proteins as well.

Much public interest in the fields of biotechnology and evolution focuses on DNA and its role as the molecular code of life. A complete understanding of DNA and inheritance, however, requires a greater appreciation of the importance of proteins. DNA dictates the production of specific proteins, but the proteins themselves directly determine an organism's traits. The passing of

DNA mutations—and thus, alterations in protein sequences—to offspring helps bring about speciation, the creation of distinct new species from a common ancestor. Therefore, a close study and comparison of particular proteins from different species may indicate how closely related the species are.

Species that diverged from a common ancestor a long time ago are less similar biochemically than those that diverged more recently. The biochemical composition of organisms includes their protein molecules. Thus, the degree of relatedness of two species can be estimated from the amount of similarity between their protein makeups. To compare protein profiles between organisms, scientists separate the mixture of protein molecules in a particular tissue (such as muscle tissue) by gel electrophoresis. This creates a unique pattern of bands for each organism, called a protein fingerprint. The individual bands correspond to different proteins and may vary in intensity between species. In addition, some bands (i.e., proteins) may be visible in one species fingerprint but not in another. In general, protein fingerprint patterns obtained from different species are more similar when the species are more closely related and less similar when they are more distantly related.

Although gel electrophoresis of proteins and the resulting protein fingerprints can provide general information about the protein profiles and the relatedness of different species, it does not supply any specific information about the actual composition of those proteins. Analysis and comparison of the amino acid sequence of the individual proteins provides the more-detailed information from which evolutionary relationships can be reconstructed.

To carry out such analysis, scientists assemble the amino acid sequence of a single protein or a group of proteins from the species in question. They then count the number of amino acid differences between the protein sequences from the two species. The more differences, the longer ago the two species diverged. For example, the amino acid sequence of the protein cytochrome c is identical in humans and chimpanzees. There is only one difference between the cytochrome c sequences of humans and rhesus monkeys. On an evolutionary scale, humans and rhesus monkeys diverged relatively recently. In contrast, there are 13 differences between humans and dogs, 20 differences between humans and rattlesnakes, and 31 differences between humans and tuna. Humans and these species diverged farther back in time. Scientists use this comparative information along with protein fingerprint patterns and data from the fossil record to estimate when lineages leading to various modern species diverged from a common ancestor.

Fish Muscle Proteins

Fish represent a diverse group of organisms that have evolved to live in many different aquatic environments. The evolution of different groups of fish and the varying degrees to which they are related are topics of ongoing study. Students will compare the protein fingerprints of seven different types of fish through gel electrophoresis of the fish protein extracts provided. Fish were chosen as the sample sources because there are many different varieties and because protein sources for many fish species are readily available. This manual includes an Evolutionary Tree of Fish to aid interpretation and analysis of the fish protein fingerprint patterns.

The proteins samples provided were extracted from fish muscle tissue. There are a number of proteins that make up muscle tissue. Following is a list of some muscle proteins that may be present as bands in the fish protein fingerprints, along with their approximate molecular weights in kilodaltons: actin (42 kDa), myosin heavy chain (210 kDa), myosin light chains (15, 17, and 24 kDa), titin (3000 kDa), dystrophin (400 kDa), filamin (270 kDa), spectrin (265 kDa), nebulin (107 kDa), α -actinin (100 kDa), gelsolin (90 kDa), fimbrin (68 kDa), tropomyosin (35 kDa), troponin T (30 kDa), thymosin (5 kDa). Since all of the fish protein samples are from muscle tissue, there will be some expected similarities. Nevertheless, difference in the protein banding patterns will also be apparent and these differences can be used to assess evolutionary relatedness.

Gel Electrophoresis of Proteins

In gel electrophoresis, separation of charged molecules is achieved by subjecting them to an electric current which forces them to migrate through a gel matrix. The behavior of a molecule during gel electrophoresis depends on its size, shape, and net charge. Linear DNA molecules have uniformly negatively charged backbones and a shape that normally varies only in its length. Therefore, migration of DNA is directly dependent on the size of the DNA fragment. The migration of proteins, however, is affected by multiple factors involving their structural organization.

There are four levels of structural organization in proteins. The primary structure of a protein is its sequence of amino acids. Amino acids can be positively charged, negatively charged, or neutral. This means that proteins can carry either a net positive, net negative, or neutral charge depending on the combination of amino acids they contain.

The shapes of proteins vary widely. The shape of a protein is created by its secondary, tertiary, and quaternary structure. In secondary protein structure, hydrogen bonds form between adjacent parts of the amino acid chain to form folded, coiled, or twisted shapes, including α -helices and β -sheets. Additional interactions including hydrogen bonds, hydrophobic interactions, electrostatic interactions, and/or disulfide bonds lead to the tertiary structure of a protein. At the quaternary structural level, several folded amino acid chains associate in unique ways to form a functional protein with a distinctive shape.

Native conformations of proteins (the form in which they are biologically active) vary widely in charge and shape. As such, the molecular weight of proteins cannot be determined by electrophoresis of native proteins. To make protein migration rates a function of molecular weight, it is necessary to impose a uniform shape and charge on all of the proteins in a mixture. This can be primarily achieved by treating the protein mixture with the negatively charged detergent sodium dodecyl sulfate (SDS) and heat. Treatment with SDS and heat disrupts hydrogen bonds and unfolds the protein structure. SDS also binds to and coats the protein backbone, regardless of the amino acid sequence, and imparts a uniform negative charge to all the molecules. Treating protein samples with a reducing agent such as β -mercaptoethanol breaks disulfide bonds and denatures the proteins into linear chains of amino acids (its primary structure). Under these conditions and for the purpose of electrophoresis, all of the proteins in a mixture assume the same shape and charge. They differ only in molecular weight. Like DNA, they migrate toward

the positive electrode during electrophoresis at a rate inversely proportional to the \log_{10} of their molecular weights.

The buffer that the fish protein extracts are provided in contains both SDS and β -mercaptoethanol to disrupt the structure of the proteins. To ensure that the proteins are fully denatured, the samples should be boiled immediately before being loaded onto the gels, as described in the procedure. To maintain protein denaturation during electrophoresis, the gels are made with a buffer that contains SDS. The electrophoresis running buffer also contains SDS.

Electrophoresis on Polyacrylamide Gels

The two gel materials most often used in molecular biology applications are agarose and polyacrylamide. Because of its greater resolving power, polyacrylamide is popularly used for protein separations. Polyacrylamide gels can separate molecules that differ in size by as little as 0.2% (1 bp in 500 if working with DNA).

Polyacrylamide is a polymer of the monomer acrylamide. In the presence of free radicals, acrylamide polymerizes into long chains that create a viscous solution of no particular use. To form a rigid gel matrix, acrylamide is polymerized in the presence of a second monomer, N,N'-methylenebisacrylamide (bisacrylamide). The bisacrylamide polymerizes along with the acrylamide and crosslinks the chains to form a rigid meshwork. The *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260) includes four pre-cast, ready-made polyacrylamide gels.

Electrophoresis on Agarose Gels

Although polyacrylamide gels offer greater resolution of protein bands than agarose gels, they are more difficult to use and usually require vertical electrophoresis chambers. As an alternative, gels can be made with fine-sieving agarose. This type of agarose offers better resolution than regular agarose while retaining the ease of use of agarose gels. It is a reasonable compromise for applications in which some resolution can be sacrificed in return for the practical advantages. The *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255) contains enough fine-sieving agarose to run six gels.

Materials

The materials provided are designed for use with the *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255) or the *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260) only. Carolina Biological Supply Company disclaims all responsibility for any other use of these materials.

Note: Upon receipt, store the fish protein extracts in a freezer (approximately -20°C). Store the protein size standard and pre-cast polyacrylamide gels in a refrigerator (approximately 4°C). All other materials may be stored at room temperature (approximately 25°C). Avoid repeated freezing and thawing of fish protein extracts. Keep fish protein extracts on ice while in use.

Materials included in *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255)

fish protein extracts, 7 samples, 150 μ L each
protein size standards, 2 tubes, 50 μ L each
fine-sieving agarose, 14 g
Tris-glycine-SDS buffer, 5 \times concentrate, 500 mL
Coomassie[®] protein stain solution, 500 mL
destain solution, 5 \times concentrate, 500 mL
latex gloves, 6 pairs
staining trays, 6
Teacher's Manual with reproducible Student Guide and Evolutionary Tree of Fish

Needed, but not supplied for *Fish Protein Fingerprinting on Agarose Gels* kit (21-1255)

horizontal gel electrophoresis chambers
gel casting trays
well-forming combs
masking tape (for sealing gel trays)
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 μ L volumes, or other gel-loading device
distilled or deionized water
containers with ice
platform shaker (optional)
water bath, 65°C (optional)
transfer pipets or Pasteur pipets (optional)
white light illuminator (optional)

Materials included in *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)

fish protein extracts, 7 samples, 150 μ L each
protein size standards, 50 μ L
pre-cast polyacrylamide gels in Tris-glycine-SDS buffer, 4
Tris-glycine-SDS buffer, 5 \times concentrate, 500 mL
Coomassie[®] protein stain solution, 500 mL
destain solution, 5 \times concentrate, 500 mL
latex gloves, 8 pairs
staining trays, 4
Teacher's Manual with reproducible Student Guide and Evolutionary Tree of Fish

Note: There is enough extra of the fish protein extracts, the stain and destain solutions, and the Tris-glycine-SDS buffer for 6 stations, should you have or choose to purchase additional gels, protein marker, staining trays, and gloves.

Needed, but not supplied for *Fish Protein Fingerprinting on Polyacrylamide Gels* kit (21-1260)

vertical gel electrophoresis chambers for 9.5 × 10-cm gels
power supplies capable of providing 130 volts
water bath, boiling
micropipets and tips capable of measuring 10 µL volumes, or other gel-loading device
distilled or deionized water
flathead screwdriver, small
containers with ice
transfer pipets or Pasteur pipets (optional)
platform shaker (optional)
white light illuminator (optional)

Teacher Tips

- Agarose gels may be cast during one lab period and stored up to two days in the electrophoresis chamber covered in 1× Tris-glycine-SDS buffer. Loading the gels with fish protein samples and performing electrophoresis can then be performed on a subsequent day.
- Store the fish protein extracts in a freezer (approximately –20°C) and do not thaw them until you are ready to use them. Repeated freezing and thawing can degrade the protein samples and decrease the quality of the protein fingerprints. Keep the extracts on ice during use.
- The fish protein samples must be heated in a boiling water bath before loading on the gel. Heat the water for the boiling water bath while students are setting up their gels, so that the water bath is ready when the loading stage is reached.
- The protein size standard is included in the electrophoresis as a control to help determine whether the gels were run properly. As an extension, you may wish to have your students use this standard to estimate the sizes of particular proteins in their samples.

Safety Tips

- Eye protection is recommended for all procedures associated with this activity. Wear gloves when handling the polyacrylamide gels.
- The Tris-glycine-SDS buffer, Coomassie® protein stain, and destain solution are nontoxic and may be disposed of down the drain.
- Coomassie® stain (composed of Coomassie® Brilliant Blue) is an efficient protein stain and therefore easily dyes skin and clothing. Wear gloves when working with this stain; avoid stain contact with skin and clothing.
- Polyacrylamide gels are made of polymers of acrylamide and bisacrylamide. Acrylamide is a dangerous neurotoxin, but it becomes harmless when polymerized with bisacrylamide to form a polyacrylamide gel. However, gels may still contain traces of unpolymerized material and should be handled with gloves. Used gels may be disposed of in the regular trash.

- The pre-cast polyacrylamide gels contain 0.02% sodium azide as a preservative to prevent microbial contamination during refrigerated storage. Sodium azide at this concentration is not known to cause health problems, although high concentrations of sodium azide are harmful (refer to the Materials Safety Data Sheet supplied for these gels by the manufacturer).

Composition of Solutions

Fish protein extracts are provided in:

50 mM Tris-HCl, pH 6.8
 5% β -mercaptoethanol
 2% sodium dodecyl sulfate
 0.1% bromphenol blue
 10% glycerol

Protein size standard

The tube(s) of protein standard provided contain approximately 1 mg/mL each of the following proteins:

Protein	Molecular weight (kilodaltons)
myosin	200.0
β -galactosidase	116.3
phosphorylase B	97.4
bovine serum albumin	66.3
glutamic dehydrogenase	55.4
lactate dehydrogenase	36.5
carbonic anhydrase	31.0
trypsin inhibitor	21.5
lysozyme	14.4
aprotinin	6.0
insulin B chain	3.5
insulin A chain	2.5

Tris-glycine-SDS buffer, 5x concentrate

0.125 M Tris base
 1.25 M glycine
 0.5% SDS (sodium dodecyl sulfate)

Coomassie® Protein Stain

10% acetic acid
 10% isopropanol
 0.25% Coomassie® Brilliant Blue

Destain Solution, 5x concentrate

50% acetic acid
 50% isopropanol

Time Requirements

Plan your activities according to the length of your lab periods. Protein extracts can be stored in the freezer for months. Gels can be left in destain solution for several days.

Activity	Approximate Time Required
Preparation of working solutions	10 min
Preparing agarose gels (if applicable)	20 min
Setting up and loading gels	30 min
Running gels	1 hr 15 min
Staining gels	Agarose: 10 min Polyacrylamide: 45 min
Destaining gels	Overnight

Pre-Lab Preparation

Note: Two different electrophoresis and staining procedures are included in the Student Guide—one for use with agarose gels and one for use with polyacrylamide gels. *Photocopy only the procedure relevant to the gels you are using.* The introduction and questions are the same regardless of which gels are used.

Photocopy the Student Guide, *including the appropriate electrophoresis and staining procedures*, and the Evolutionary Tree of Fish for each student or group of students. You may distribute the Evolutionary Tree of Fish along with the Student Guide or you may wish to withhold this information until after students have made their own predictions about the relatedness of the fish species (see the Questions section).

Station Setup

Following is a list of the materials needed for one station to perform the activities in this lab. The materials provided in the *Fish Protein Fingerprinting on Agarose Gels* kit are sufficient for running six agarose gels (6 stations). The materials provided in the *Fish Protein Fingerprinting on Polyacrylamide Gels* kit are sufficient for running four polyacrylamide gels (4 stations). **Note:** There is enough extra of the fish protein extracts, the stain and destain solutions, and the Tris-glycine-SDS buffer for 6 stations, should you have or choose to purchase additional gels, protein marker, staining trays, and gloves.

The protein size standard and seven fish protein samples must be shared among the laboratory workstations in the classroom. Divide your class size accordingly to work at the appropriate number of stations. Prepare as many setups as needed for your class.

For *Fish Protein Fingerprinting on Agarose Gels*, each station will need:

- gel casting tray
- well-forming comb
- masking tape (for sealing gel trays)
- 35 mL of prepared 4% agarose solution
- horizontal gel electrophoresis chamber
- 350 mL of prepared 1× Tris-glycine-SDS buffer

- disposable transfer pipet or Pasteur pipet (optional)
- gel loading device (e.g., micropipettor and tips)
- 10 μ L of protein size standard
- 10 μ L of each fish sample, on ice
- power supply (shared)
- staining tray
- latex gloves, 1 pair
- distilled or deionized water
- 50–75 mL of Coomassie[®] stain
- 100–150 mL of prepared 1 \times destain solution

For Fish Protein Fingerprinting on Polyacrylamide Gels, each station will need:

- latex gloves, 2 pairs
- pre-cast polyacrylamide gel
- vertical electrophoresis chamber
- 350 mL of prepared 1 \times Tris-glycine-SDS buffer
- disposable transfer pipet or Pasteur pipet (optional)
- gel loading device (e.g., micropipettor and tips)
- 10 μ L of protein size standard
- 10 μ L of each fish sample, on ice
- power supply (shared)
- flathead screwdriver, small (shared)
- staining tray
- distilled or deionized water
- 50–75 mL of Coomassie[®] stain
- 100–150 mL of prepared 1 \times destain solution

Preparation of Working Solutions

Tris-glycine-SDS buffer is supplied at a 5 \times concentration. To prepare the working 1 \times concentration, dilute the supplied Tris-glycine-SDS buffer 1:5 by adding 400 mL of distilled or deionized water to each 100 mL of 5 \times concentrate. Each station will need approximately 350 mL of 1 \times Tris-glycine-SDS buffer.

Coomassie[®] stain is supplied at a 1 \times working concentration. No dilution is necessary; use as provided. The Coomassie[®] stain solution can be collected after staining and reused several times.

Destain solution is supplied at a 5 \times concentration. To prepare the working 1 \times concentration, dilute the supplied destain solution 1:5 by adding 400 mL of water to each 100 mL of 5 \times concentrate. Each station will need approximately 100–150 mL of 1 \times destain solution.

Preparation of Agarose Gels for *Fish Protein Fingerprinting on Agarose Gels*

Prepare a 4% solution of fine-sieving agarose by adding 14 g (the entire amount) of powdered fine-sieving agarose to 350 mL of prepared 1× Tris-glycine-SDS buffer in a clean 500-mL flask or beaker. Dissolve the agarose by heating the mixture in one of the following ways:

1. Cover the flask or beaker and heat in a boiling water bath until the agarose is completely dissolved. The water level should be just above the level of the agarose mixture. To prevent the agarose from boiling over, swirl the container every few minutes during heating. Remove from heat at the first sign of vigorous boiling.
2. Heat the uncovered container on a hot plate with magnetic stirring capability. Place a magnetic stir bar in the flask/beaker and stir at a continuous, moderate rate. Heat until the agarose is completely dissolved. Remove from heat at the first sign of vigorous boiling.

The 4% solution of fine-sieving agarose has a tendency to form bubbles while heating. Microwaving increases the number of bubbles produced and is therefore not a recommended means of preparing the agarose solution.

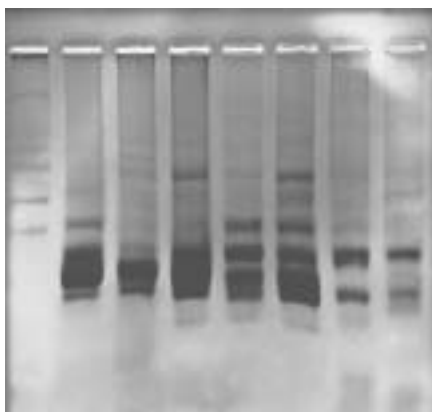
Allow the flask/beaker to cool until it can be held in a bare hand without pain. It should still feel warm and be around 65°C. You can use the agarose immediately or hold it at this temperature in a 65°C-water bath until you are ready to use it.

Procedure

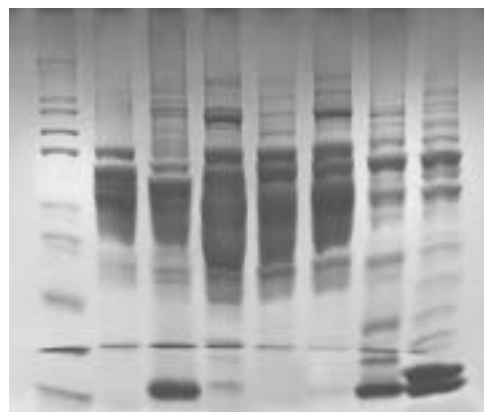
Refer to the Student Guide for step-by-step procedures for fish protein fingerprinting on agarose and polyacrylamide gels. Note that two different electrophoresis and staining procedures are included in the Student Guide—one for use with agarose gels and one for use with polyacrylamide gels. *Refer only to the procedure relevant to the gels you are using.*

Expected Results

The cover of this Teacher's Manual shows color photographs of expected results for fish protein fingerprinting on agarose gels and on polyacrylamide gels. These results are also reproduced below.



Agarose gel



Polyacrylamide gel

Answers to Questions in the Student Guide

1. Before looking at the Evolutionary Tree of Fish, compare the protein profiles from the seven fish species. On the basis of these banding patterns, determine which species you think are most closely and most distantly related. Explain your reasoning. It may be helpful to focus on the less intense bands in the protein fingerprints.

Answers will vary.

2. Compare your hypothesis on the relatedness of the fish to the Evolutionary Tree of Fish. How closely did your analysis match this relational tree?

Answers will vary.

3. What aspects of **native** proteins affect their rate of migration during gel electrophoresis?

Migration rates are affected by the size, shape, and net charge of the proteins. Proteins can have an overall positive, negative, or neutral charge. Protein sizes vary widely and their structural organization results in unique three-dimensional conformations.

4. How do scientists make the migration rates of proteins reflect their molecular weights (sizes) and not their charges or shapes?

A uniform shape and charge is imposed on all of the proteins being analyzed. Treatment with the detergent SDS gives the proteins a uniformly negative charge and unfolds their structure. Heat and reducing agents such as β -mercaptoethanol also help denature proteins to their primary structure. Once in their primary structure, for the purpose of electrophoresis, proteins have the same shape. Once the proteins are effectively all the same charge and shape, they will run according to their molecular weights.

References

Printed Resources

For more information on protein structure and its relationship to function, consult a reference such as *Recombinant DNA and Biotechnology: A Guide for Teachers* by Kreuzer and Massey (American Society for Microbiology, 2001; Carolina Biological Supply item RN-21-2218).

Carroll, R.L. 1988. *Vertebrate Paleontology and Evolution*. W.H. Freeman & Co., New York.

Nelson, J.S. 1994. *Fishes of the World*, 3rd ed. John Wiley and Sons Inc., New York.

Online Resources

At the time of this printing, the web sites listed below are active. You may wish to perform an independent search for related sites.

Regulatory Fish Encyclopedia. <http://vm.cfsan.fda.gov/~frf/rfe0.html>

Tree of Life. <http://phylogeny.arizona.edu/tree/phylogeny.html>

Fish Protein Fingerprinting on Agarose and Polyacrylamide Gels

Introduction

Species that diverged from a common ancestor a long time ago are less similar biochemically than those that diverged more recently. The biochemical composition of organisms includes their protein molecules. Thus, the degree of relatedness of two species can be estimated from the amount of similarity between their protein makeups. To compare protein profiles between organisms, scientists separate the mixture of protein molecules in a particular tissue (such as muscle tissue) by gel electrophoresis. This creates a unique pattern of bands for each organism, called a protein fingerprint. The individual bands correspond to different proteins and may vary in intensity between species. In addition, some bands (i.e., proteins) may be visible in one species fingerprint but not in another. In general, protein fingerprint patterns obtained from different species are more similar when the species are more closely related and less similar when they are more distantly related.

Fish represent a diverse group of organisms that have evolved to live in many different aquatic environments. The evolution of different groups of fish and the varying degrees to which they are related are topics of ongoing study. In this exercise, you will compare the protein fingerprints of seven different types of fish through gel electrophoresis of the fish protein extracts provided. Fish were chosen as the sample sources because there are many different varieties and because protein sources for many fish species are readily available.

The behavior of a molecule during gel electrophoresis depends on its size, shape, and net charge. Linear DNA molecules have uniformly negatively charged backbones and a shape that normally varies only in its length. Therefore, migration of DNA is directly dependent on the size of the DNA fragment. The migration of proteins, however, is affected by multiple factors involving their structural organization.

There are four levels of structural organization in proteins. The primary structure of a protein is its sequence of amino acids. Amino acids can be positively charged, negatively charged, or neutral. This means that proteins can carry either a net positive, net negative, or neutral charge depending on the combination of amino acids they contain.

The shapes of proteins vary widely. The shape of a protein is created by its secondary, tertiary, and quaternary structure. In secondary protein structure, hydrogen bonds form between adjacent parts of the amino acid chain to form folded, coiled, or twisted shapes, including α -helices and β -sheets. Additional interactions, such as hydrogen bonds, hydrophobic interactions, electrostatic interactions, and/or disulfide bonds lead to the tertiary structure of a protein. At the quaternary structural level, several folded amino acid chains associate in unique ways to form a functional protein with a distinctive shape.

Native conformations of proteins (the form in which they are biologically active) vary widely in charge and shape. As such, the molecular weight of proteins cannot be determined by electrophoresis of native proteins. To make protein migration rates a function of molecular weight, it is necessary to impose a uniform shape and charge on all of the proteins in a mixture. This can be primarily achieved by treating the protein mixture with the negatively charged detergent sodium dodecyl sulfate (SDS) and heat. Treatment with SDS and heat disrupts hydrogen bonds and unfolds the protein structure. SDS also binds to and coats the protein backbone, regardless of the amino acid sequence, and imparts a uniform negative charge to all the molecules. Treating protein samples with a reducing agent such as β -mercaptoethanol breaks disulfide bonds and denatures the proteins into linear chains of amino acids (its primary structure).

Under these conditions and for the purpose of electrophoresis, all of the proteins in a mixture assume the same shape and charge. They differ only in molecular weight. Like DNA, they migrate toward the positive electrode during electrophoresis at a rate inversely proportional to the \log_{10} of their molecular weights.

The buffer that the fish protein extracts are provided in contains both SDS and β -mercaptoethanol to disrupt the structure of the proteins. To ensure that the proteins are fully denatured, the samples should be boiled immediately before being loaded onto the gels, as described in the procedure. To maintain protein denaturation during electrophoresis, the gels are made with a buffer that contains SDS. The electrophoresis running buffer also contains SDS.

Electrophoresis Procedure for Fish Protein Fingerprinting on Agarose Gels

These instructions are written for use with the Carolina™ Gel Electrophoresis Chamber (21-3668). If you do not have this equipment, modify these instructions to suit your apparatus.

1. Seal the open ends of the gel casting tray with masking tape so that no seams or gaps appear. Insert the well-forming comb in the top set of grooves over the black stripe in the casting tray.
2. Carefully pour a thin layer of the prepared 4% fine-sieving agarose solution into the casting tray until it just covers the bottom of the tray (~35 mL). Thin gels will give more desirable protein electrophoresis results than thick gels.
3. While the agarose is still liquid, move bubbles and debris to the perimeter of the tray with the well-forming comb. Return the comb to its position in the top set of grooves in the casting tray.
4. Allow the gel to sit undisturbed while it solidifies. Be careful not to move or jar the casting tray during this time. Gel solidification will occur within 10 minutes.
5. Once the agarose has solidified, slowly and carefully remove the comb from the gel without tearing the wells. Remove the tape at the ends of the casting tray to unseal the gel. Place the gel and casting tray into the electrophoresis chamber oriented with the red stripe towards the positive (red) end, and the black stripe towards the negative (black) end.
6. Fill the electrophoresis chamber with 1× Tris-glycine-SDS buffer to a level that just covers the surface of the gel.
7. The gel is now ready to load with samples. If you will be loading the gel at another time, cover the electrophoresis tank with the lid to prevent the gel from drying out.
8. Rinse gel debris from the wells of the gel by pipetting the surrounding 1× Tris-glycine-SDS buffer in and out of the wells. If available, a disposable transfer pipet or a Pasteur pipet works well for this purpose.
9. Prepare fish protein extracts for loading by immersing the sample-containing portion of the tubes in a boiling water bath for 3 to 5 minutes. Do not immerse the lids. **Do not boil the protein size standard.** Immediately after the samples have been heated, load them according to the following instructions.
10. Load 10 µL of protein size standard and fish extracts into the wells (also called lanes) from left to right following the order and procedure below.

To load the first sample (protein size standard) into the well, draw 10 µL of the sample into a pipet tip. Using your dominant hand, steady the pipet over the well. Rest the elbow of your dominant arm on the lab bench to stabilize your hand. Using your non-dominant hand, guide the pipet tip through the surface of the buffer and position it directly over the well. Slowly expel the sample into the well (see Figure 1). The sample will sink to the bottom of the well because it has been mixed with glycerol to increase its density. Repeat this process for each sample, continuing from left to right according to the Order of Loading. Use a clean pipet tip for each sample. Be sure to check the label on each tube before you load to ensure that it matches the intended order.

Order of Loading

lane 1	protein size standard
lane 2	shark
lane 3	catfish
lane 4	salmon
lane 5	swordfish
lane 6	tuna
lane 7	flounder
lane 8	orange roughy

11. Connect the electrodes to the power supply [positive lead to positive input (red to red) and negative lead to negative input (black to black)] and run the gel at 130 volts. At this voltage, the bromphenol blue loading dye in the samples should move through the gel to the bottom in approximately 1 hour and 15 min.
12. After electrophoresis is complete, turn off the power supply and remove the lid of the electrophoresis chamber.



Figure 1. Loading samples into the agarose gel.

Staining and Destaining Procedure for Agarose Gels

1. Wearing gloves, place the gel in a staining tray and flood it with Coomassie® stain solution until it is completely covered (~50 to 75 mL). The entire gel will turn blue. Let the gel stain for 10 minutes.
2. After staining is complete, carefully return the Coomassie® stain to its container. The Coomassie® stain solution can be reused several times.
3. Rinse the gel several times with distilled or deionized water by repeatedly flooding the tray with water and pouring the wash down the drain.
4. Flood the gel with destain solution until it is completely immersed (~50 to 75 mL). If possible, gently agitate the gel on a platform shaker while it destains. Allow the gels to destain overnight. The destain solution can be changed during destaining, to facilitate the destaining process. Distinct blue protein banding patterns should become visible in the gel as the blue background lightens. Gels can be stored covered in destain solution for several days.
5. Gels are best viewed when placed on a white light illuminator, if available.

Electrophoresis Procedure for Fish Protein Fingerprinting on Polyacrylamide Gels

These instructions are written for use with the Carolina™ Vertical Gel Electrophoresis Chamber (21-3671). If you do not have this equipment, modify these instructions to suit your apparatus.

1. Wearing gloves, remove the polyacrylamide gel from its aluminum package. Rinse the gel with distilled or deionized water.
2. Remove the comb and rinse the exposed wells with distilled or deionized water.
3. Inspect the bottom of your gel cassette. If the bottom of the gel is exposed, proceed to the next step. If a plastic tab conceals the bottom, snap off the removable lower portion of the plastic cassette from the pre-cast gel (there is an indentation that separates the bottom tab from the main unit). Rest the cassette on the bench top with the bottom tab overhanging the edge. Press downward on the cassette with one hand, then rotate the detachable tab up and down until it snaps free (see Figure 1). Removing the lower portion of the cassette exposes the bottom of the gel so that it can be in direct contact with the running buffer during electrophoresis.
4. Place the pre-cast gel in the lower electrophoresis chamber with the notched plate flush against the side of the upper buffer chamber. The edges of the gel should rest on small plastic platforms that raise the gel about 1 mm off the bottom of the chamber. This will allow the running buffer to contact the bottom of the gel during electrophoresis (see Figure 2).
5. Add just enough 1× Tris-glycine-SDS buffer to the lower chamber to cover the bottom of the gel. Make sure no air bubbles are trapped beneath the gel as they will interfere with the electrical current during electrophoresis.
6. Secure the gel in place by sliding the lid (with the red electrical lead) on the lower chamber and tightening the thumbscrews all the way. If the screws are not securely fastened, buffer will leak from the upper chamber to the lower chamber and electrophoresis will be halted.
7. Add 1× Tris-glycine-SDS buffer to the upper chamber until the top of the gel is covered. The buffer layer should remain at the same level. If the buffer level begins to sink below the top of the gel, tighten the thumbscrews and add more buffer to the upper chamber. The top and bottom of the gel must be covered with buffer at all times for an electrical current to be maintained.

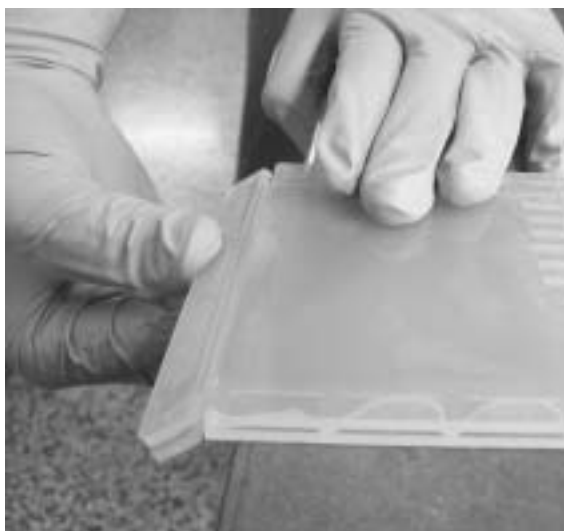


Figure 1. Removing the lower portion of the plastic cassette.

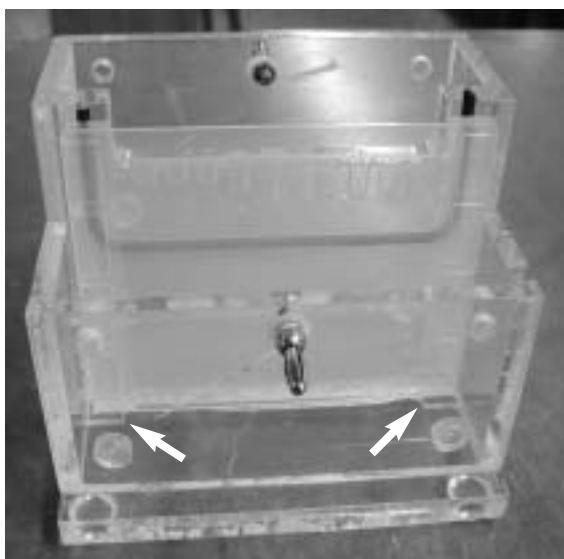


Figure 2. Placement of the polyacrylamide gel in the electrophoresis chamber (arrows indicate the raised platforms on which the edges of the gel should rest).

8. Slide the lid (with the black electrical lead) onto the upper chamber and tighten the thumbscrews to secure it in place.
9. Rinse gel debris from the wells of the gel by pipetting the surrounding 1× Tris-glycine-SDS buffer in and out of the wells. If available, a disposable transfer pipet or a Pasteur pipet works well for this purpose.
10. Prepare fish protein extracts for loading by immersing the sample-containing portion of the tubes in a boiling water bath for 3 to 5 minutes. Do not immerse the lids. **Do not boil the protein size standard.** Immediately after the samples have been heated, load them according to the following instructions.
11. Load 10 µL of protein size standard and fish extracts into the wells (also called lanes) from left to right following the order and procedure listed below.

To load the first sample (protein size standard) into the well, draw 10 µL of the sample into a pipet tip. Coming from the upper chamber, place the tip of the loading device against the unnotched gel plate directly over the well to be loaded. Slowly expel the sample into the well (see Figure 3). The sample will sink to the bottom of the well because it has been mixed with glycerol to increase its density. Repeat this process for each sample, continuing from left to right in the order given below. Use a clean pipet tip for each sample. Be sure to check the label on each tube before you load to ensure that it matches the intended order.

Order of Loading

lane 1	protein size standard
lane 2	shark
lane 3	catfish
lane 4	salmon
lane 5	swordfish
lane 6	tuna
lane 7	flounder
lane 8	orange roughy

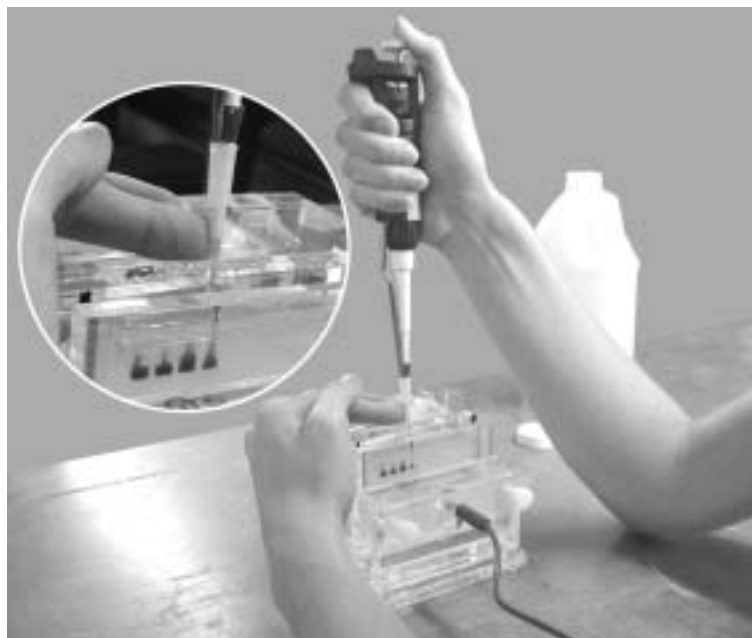


Figure 3. Loading samples into the polyacrylamide gel.

12. Connect the electrodes to the power supply [positive lead to positive input (red to red) and negative lead to negative input (black to black)] and run the gel at 130 volts. At this voltage, the bromphenol blue loading dye in the samples should move through the gel to the bottom in approximately 1 hour and 15 min.
13. After electrophoresis is complete, turn off the power supply and remove the lid to the bottom chamber by loosening the thumbscrews and sliding off the lid.
14. Wearing gloves, take the gel cassette out of the chamber. The polyacrylamide gel must now be removed from the plastic cassette. To remove the gel, first place the cassette on a lab bench. Insert the tip of a small flat screwdriver between the plates and twist the screwdriver gently until you hear the seal break (see Figure 4). Move along the edge of the cassette and repeat this action until the entire seal is broken. Break the seal on the other side in the same manner. Gently remove the top plate to access the gel.

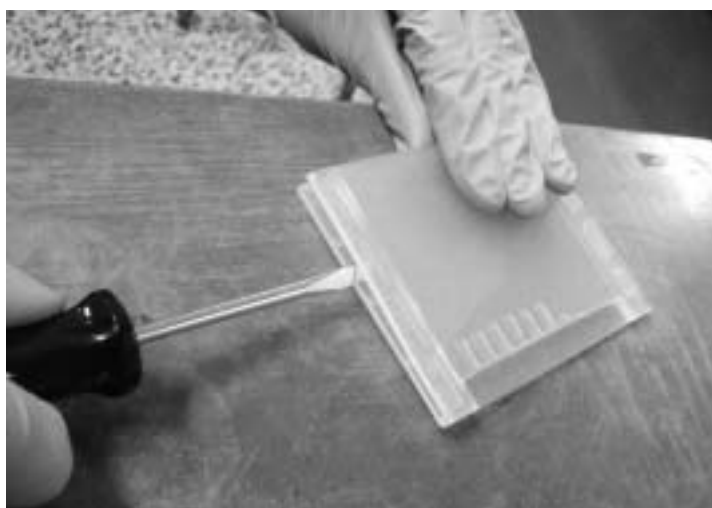


Figure 4. Removing the gel from the plastic cassette.

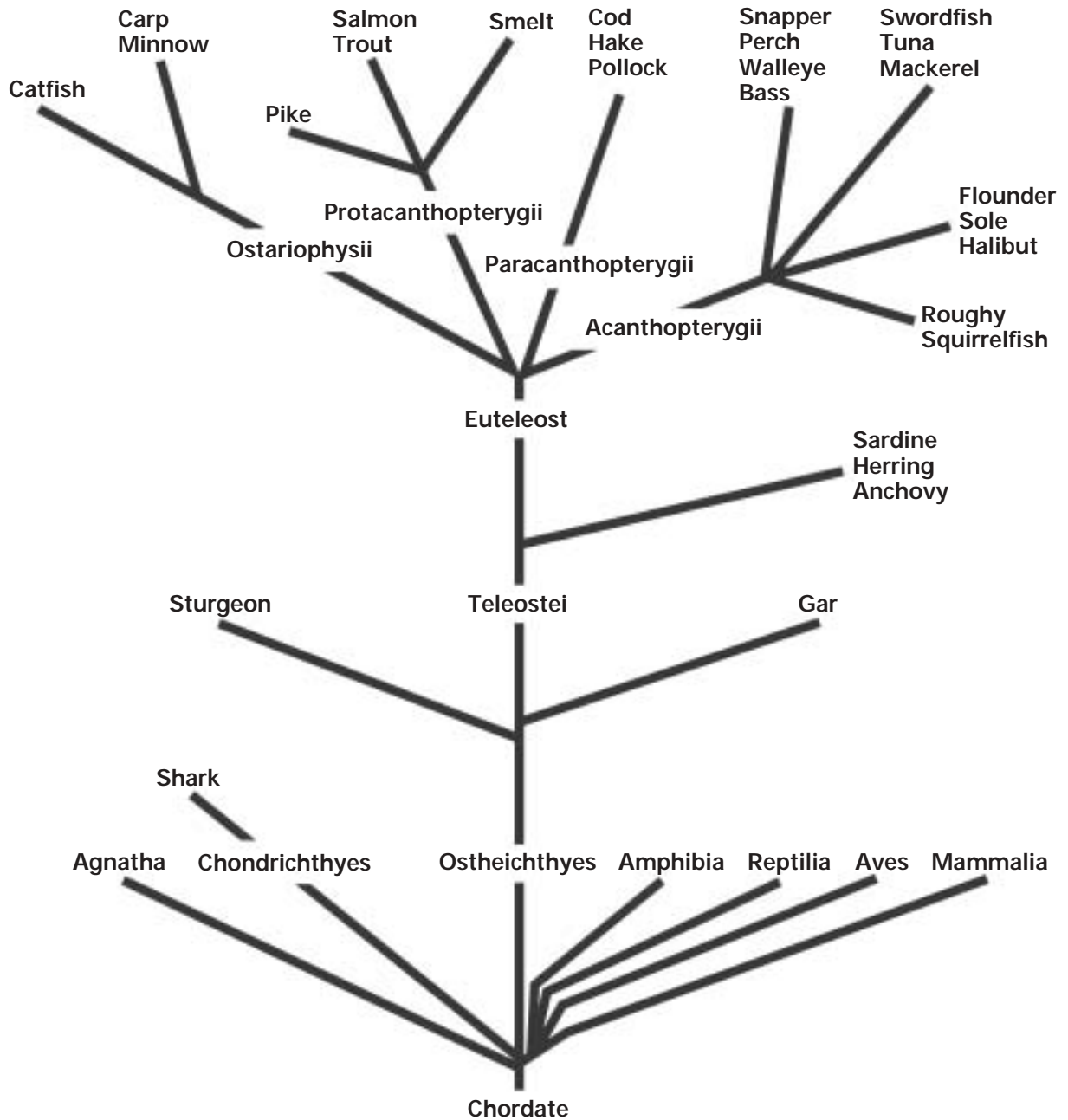
Staining and Destaining Procedure for Polyacrylamide Gels

1. Wearing gloves, place the gel in a staining tray and flood it with Coomassie® stain solution until it is completely covered (~50 to 75 mL). The entire gel will turn blue. Let the gel stain for 45 minutes.
2. After staining is complete, carefully return the Coomassie® stain to its container. The Coomassie® stain solution can be reused several times.
3. Rinse the gel several times with distilled or deionized water by repeatedly flooding the tray with water and pouring the wash down the drain.
4. Flood the gel with destain solution until it is completely immersed (~50 to 75 mL). If possible, gently agitate the gel on a platform shaker while it destains. Allow the gels to destain overnight. The destain solution can be changed during destaining, to facilitate the destaining process. Distinct blue protein banding patterns should become visible in the gel as the blue background lightens. Gels can be stored covered in destain solution for several days.
5. Gels are best viewed when placed on a white light illuminator, if available.

Questions

1. Before looking at the Evolutionary Tree of Fish, compare the protein profiles from the seven fish species. On the basis of these banding patterns, determine which species you think are most closely and most distantly related. Explain your reasoning. It may be helpful to focus on the less intense bands in the protein fingerprints.
2. Compare your hypothesis on the relatedness of the fish to the Evolutionary Tree of Fish. How closely did your analysis match this relational tree?
3. What aspects of **native** proteins affect their rate of migration during gel electrophoresis?
4. How do scientists make the migration rates of proteins reflect their molecular weights (sizes) and not their charges or shapes?

Evolutionary Tree of Fish



Note: This is a partial representation of the evolutionary tree of fish, for comparison only (information from Internet sites: Ichthyology Web Resources, Tree of Life, and European Register of Marine Species).

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