

# Proteins and Clades: A Lab Exercise Using Molecular Methods to Illustrate Phylogenetic Relationships Among Fish

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## Extended Abstract

Traditionally, morphology has been the most widely used tool to study the evolutionary relationships among organisms living on this planet; however, morphological characteristics do not always accurately reflect true evolutionary relationships. With recent advances in molecular biology, scientists have realized that comparison of DNA sequences between organisms can provide a wealth of information about their phylogenies, allowing for predictions of evolutionary relationships. With the advent of cheap and accessible whole genome sequences over the last decade, the prevalence of these studies has grown and molecular phylogenetics has become an increasingly important field.

As an alternative to DNA analysis, protein analysis can also be used to examine evolutionary relationships (Brown 2002). Denaturing protein gel electrophoresis can be used to compare the electrophoretic mobilities and molecular masses of proteins from different organisms, which can serve as a means of assessing the degree of similarity of the proteins from various organisms. This type of protein analysis is not as sensitive as DNA sequence analysis because only significant differences in protein size and sequence can be detected, but it is technically simpler and still provides information useful for comparing phylogenetic relationships among closely-related species. This lab exercise uses protein gel electrophoresis to compare protein profiles between different species of fish, and uses the results to infer the phylogenetic relationships among them. This experiment is conducted as part of the laboratory component of the Zoology 400 (*Aquatic Vertebrates*) course in the Ecology/Environmental Biology stream of the Biology degree program at MacEwan University. Most students in the course have had very little exposure to molecular biology techniques and are primarily familiar with the morphological characteristics of the fish. This exercise provides them with a hands-on opportunity to gather data using molecular techniques, compare their results with morphological evidence, and incorporate this information into their understanding of the evolutionary relationships among major groups of fishes.

The design of this lab exercise was originally based on a Bio-Rad Comparative Proteomics kit (Bio-Rad, 2011). This kit had students use pre-cast gels and prepared materials, resulting in minimal student engagement in the molecular aspects of the experiment. The procedure was modified to allow students to perform the gel preparation and gel electrophoresis themselves, providing these students with valuable experience in molecular lab techniques. This hands-on experience also better equipped the students to explain potential sources of experimental error that could affect the accuracy of their cladograms. Students prepared a discontinuous SDS-polyacrylamide gel (4% acrylamide stacking gel, 15% resolving gel) using the Bio-Rad Protean 3 gel casting apparatus, and also prepared Tris-Glycine-SDS Running Buffer. Prior to the lab, the instructor and lab technician dissected several fish specimens to obtain muscle samples. Laemmli sample buffer (150 mM Tris-HCl pH 6.8, 2.5% SDS, 12%  $\beta$ -mercaptoethanol, 24 % glycerol, 0.01% bromophenol blue) was added to the muscle samples and mixed by vigorous vortexing for 2 minutes. The supernatant liquid containing extracted muscle proteins was then removed to a new tube, which was provided to the students.

Students were provided with these muscle protein extracts as 'unknown' samples. The samples were heated at 95°C for 5 minutes to denature proteins, and were loaded onto the prepared SDS-polyacrylamide gel alongside a ColorPlus Prestained Broad Range Protein Marker (NEB) and a purified actin-myosin standard (Bio-Rad). The samples were separated by electrophoresis in the prepared Tris-Glycine-SDS Running Buffer at 200 V in a Bio-Rad Protean 3 gel electrophoresis apparatus until the bromophenol blue dye had trav-

eled at least 2/3 of the distance through the resolving gel (approximately 45 minutes). The students disassembled the gel, transferred it to Coomassie Brilliant Blue Protein Stain (10% acetic acid, 50% methanol, 0.1% Coomassie Brilliant Blue R250), and stained with agitation at room temperature for 1 hour. Gels were then destained in 10% acetic acid, 45% methanol with agitation at room temperature overnight. Gels were photographed the next morning by students using an Automated Biospectrum Imaging System with white light transillumination.

The following week, students conducted a full analysis of their gels and prepared a cladogram based on the results. First, students prepared a standard curve of migration distance (in mm) versus molecular mass (in kDa) using semi-log graph paper by measuring the migration distances from the top of the resolving gel of each band in the molecular mass marker lane. This standard curve was then used to determine the molecular mass of each major band present in each sample lane on the gel. During these measurements, students were instructed to conduct their measurements in a reproducible manner to the nearest 1 mm. Once the molecular mass of each major protein band was determined, students created a pairwise similarity matrix to determine how many of these bands were shared between each pair of 'unknown' fish species. The presence of common bands was used as an indicator of similarity between species, allowing the students to construct a cladogram representing the relationships among the fish. Only once the students had prepared their cladograms were they provided with the true identities and relationship of the fish samples for comparison to their results.

In general, the major protein bands observed in the student-generated SDS-PAGE gels were found to exhibit differences that were more pronounced between more distantly-related fish species. The accuracy with which the students translated these patterns into a representation of the actual relationship between species depended on a variety of experimental factors including pouring a uniform polyacrylamide gel, even loading of equivalent amount of sample in each lane, even separation of proteins in the gel during electrophoresis, consistent identification of all major bands in each lane, accurate measurement of migration distances, and correct determination of molecular masses of protein bands based on the standard curve. In addition, the analysis is based on the assumption that biochemical differences in fish muscle proteins always correctly reflect evolutionary relationships.

Because so many experimental variables affect the preparation of the cladogram, student cladograms often differed from the actual phylogenetic relationship. Students usually obtained mostly correct groupings, with one or two key discrepancies from the actual cladogram. Rather than posing a problem, these discrepancies provide an excellent learning opportunity for students. As part of a formal lab report on this exercise, students were asked to compare their experimentally-derived cladogram to the 'real' cladogram reflecting established relationships. To explain the discrepancies, students needed to evaluate the validity of their experimental results and propose an explanation of how their experimental design could have affected their interpretation. These additional requirements address higher-level learning outcomes and necessitate critical thinking. This resulted in increased understanding of the molecular methods, and how these methods are connected to our understanding of phylogenetic relationships.

The students in this course have been overwhelmingly enthusiastic about this lab, and take personal pride in their newly-acquired abilities in molecular lab techniques. Their performances in lab discussions and lab reports demonstrate that this lab is successful in helping the students to integrate this new knowledge and apply it to analyze experimental results. This interdisciplinary lab experience, therefore, enables students to integrate biological principles from a variety of disciplines into a 'big picture' of fish biology and phylogeny.

**Keywords:** phylogeny of fish, clades, proteomics

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### Literature Cited

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