Proteins and Clades: A lab exercise using molecular methods to illustrate phylogenetic relationships among fish

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Introduction

Since the publication of Darwin's book on natural selection in 1859, it has been the goal of many biologists to understand the evolutionary relationships of the organisms living on this planet. Until recently, morphology has been the most widely used tool to study those relationships; 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. Evolutionary biologists are now able to study differences in the nucleotide sequence between genomes of different organisms and draw inferences about how recently they shared a common ancestor, 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 more simple and still provides information useful for comparing phylogenetic relationships among closely-related species.

The lab exercise described here uses protein gel electrophoresis to compare protein profiles between different species of fish, and uses the results to infer the phylogenetic relationships among the fish. This exercise is conducted as part of the laboratory component of the ZOOLOGY 400 (Aquatic Vertebrates) course in the Ecology/Environmental Biology stream of our Biology degree program at MacEwan University. Most students in the course generally 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 of major groups of fishes.

Learning Objectives

At the end of this lab exercise, students should be able to …

- Explain why molecular phylogenetics is an important field.
- Describe how analysis of biological molecules can be used to determine phylogenetic relationships.
- Use molecular data to prepare simple cladograms.
- Evaluate the validity of experimentally-based cladograms, and analyze experimental factors that can affect results.

Experimental Methods

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 were provided with a series of protein muscle extracts from 'unknown' fish species. Following separation of the proteins by SDS-polyacrylamide gel electrophoresis (PAGE), students determined the molecular masses of the major bands present in each lane on the gel. 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 species. Only once the students had prepared their cladograms were they provided with the true identities and relationship of the fish samples.

Advance Sample Preparation :
- Dissect fish to obtain muscle sample
- Add Laemmli sample buffer (contains SDS and reducing agent)
- Mix by vortexing to extract proteins
- Transfer extract to new tube

Gel preparation (Lab 1):
- Assemble Bio-Rad Protean 3 gel casting apparatus
- Pour a discontinuous SDS-polyacrylamide gel (4% acrylamide stacking gel, 15% resolving gel)

Student Sample Preparation (Lab 1):
- Fish muscle extracts provided to students as 'unknown' samples
- Heat at 95°C for 5 minutes
- Load onto prepared SDS-PAGE gel

Gel Electrophoresis (Lab 1):
- Fish samples (up to 8) are loaded alongside a molecular mass marker and a protein standard containing purified actin and myosin
- Gels are run in SDS-Tris-Glycine running buffer at 200 V until the dye front passes at least 2/3 of the way down the resolving gel
- Gels are stained with Coomassie Blue R250 and destained overnight in 10% acetic acid, 45% methanol

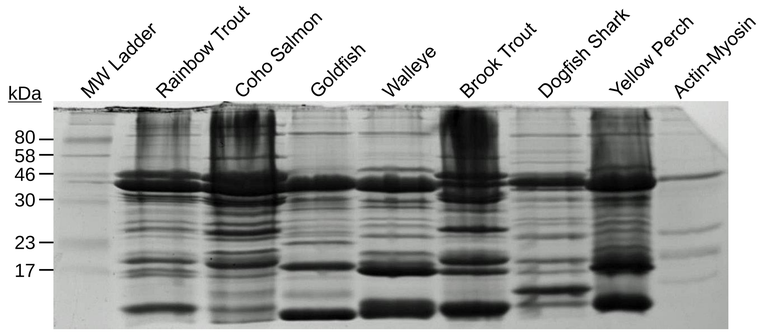

Figure 1: SDS-polyacrylamide gel, stained with Coomassie Blue R250, showing separation of total muscle proteins from fish samples. Standards for comparison are the NEB ColorPlus Prestained Broad Range Protein Markers (MW Ladder) and Bio-Rad purified rabbit actin and myosin standard (Actin-Myosin).

Gel Analysis (Lab 2):
- Migration distance measured for bands in marker and samples
- Standard curve of log molecular mass vs. migration distance used to determine molecular masses of primary bands in fish muscle samples
- Similarity matrix prepared based on the number of band similarities between each pair of fish

Predicting Relationships (Lab 2):
- Similarity matrix used to identify fish as closely or distantly related
- Cladogram prepared for unknown fish samples based on molecular data
- At the end of lab, students are provided with identities of fish samples and the 'real' cladogram based on known phylogenetic relationships

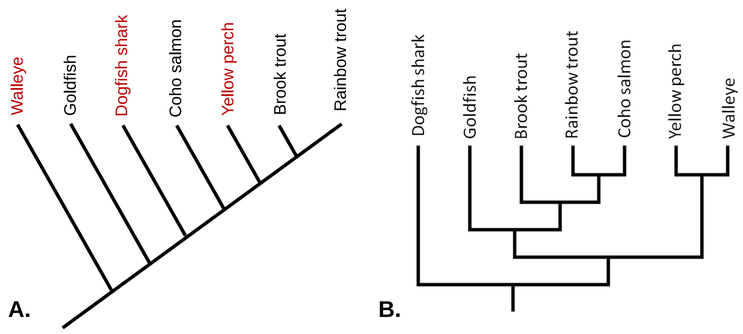

Figure 2: Cladograms representing relationships among fish species. **A**: Sample student cladogram based on experimental data. **B**: Correct cladogram representing known phylogenetic relationships. Major discrepancies in the student cladogram are highlighted in red.

Student Results

The major protein bands observed in student-generated SDS-PAGE gels (example shown in Figure 1) exhibit differences that are more pronounced between more distantly-related fish species. The accuracy with which the student translates these patterns into a representation of the actual relationship between species depends on:

- 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.
- Correct determination of molecular masses of protein bands based on a standard curve graph.
- Biochemical differences in fish muscle proteins that correctly reflect evolutionary relationships.

Because so many variables affect the preparation of the cladogram, student cladograms often differ from the actual phylogenetic relationship (Figure 2). Students usually obtain 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 discussed below.

Assessment of Learning

Students are required to present their experiment and results in a paper format (introduction, methods, results and discussion) lab report. In addition to the standard requirements to a formal lab report, which these students are introduced to in junior-level courses, the students were asked to:

- Compare their experimentally-derived cladogram to the 'real' cladogram reflecting established relationships.
- Evaluate the validity of their experimental results.
- Propose explanations of how their experimental design could have affected the validity of their results.

These additional requirements address higher-level learning outcomes and necessitate critical thinking. To adequately discuss these items, students must apply their knowledge of how the molecular methods work to analyze potential weaknesses in their methods and to explain discrepancies in their results. This results in increased understanding of the molecular methods, and how these methods are connected to our understanding of phylogenetic relationships.

Conclusions

This lab exercise introduces students to the principles of molecular phylogenetics, and assists them in applying these concepts to their current understanding of evolution in fishes. 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 exercise also has the potential to be extended to incorporate even more student involvement and critical thinking, depending on the level of the students and the resources and time available:

- Students could select their own samples, and research the real phylogenetic relationships to compare their experimental results.
- Students could dissect the fish and prepare the muscle extracts from known or unknown fish samples.
- The SDS-PAGE gel could be examined by Western analysis with an anti-myosin antibody to illustrate the difference between general protein profiles and examining the properties of a specific protein.

References

Bio-Rad. 2011. Bio-Rad Comparative Proteomics Kit I: Protein Profiler Module.

Brown T. 2002. Genomes, 2nd edition. Oxford: Wiley-Liss.