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Evo-Devo: Does Seed Protein Biochemistry Reflect Plant Phylogeny?

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Biochemistry of plant seeds should provide insights into evolution. Based on this theme, our workshop used PAGE technique to analyze seed protein extracts from two major plant families. Though the workshop focused on the methodology, the concluding message was to re-examine traditional approaches to the taxonomy of plants, by analysis of protein profiles. During the hour, participants performed abridged hands-on learning, wherein they prepared plant extracts, viewed previous gel runs and photographed protein banding of two families (*Brassicaceae* and *Fabaceae*). This exercise could provide a useful primer for an undergraduate class to determine whether seed protein patterns corroborate the relatedness of members of these selected plant families.

Keywords: seed protein electrphoresis, plant classification, developmental biology

Introduction

Developing a laboratory exercise to present an important concept and simultaneously motivate students can be daunting. Though the concept may be central to biology, the framework being modeled may present problems. Students may not have training or inclination to use a model because of lack of familiarity or they have some inherent reluctance to pursue it. For example, if the concept is cladistics, a model system using various strains of mice may not work, if the students are not trained to handle rodents or if their cost is prohibitive. In this exercise, we introduce the concept of phylogenetic relationships and taxonomy to an undergraduate class. The model system to illustrate the concept is plant seeds. Plants are easily accessible, inexpensive, and not likely to generate ethical conflicts. Most students are aware of plants as a part of their diet, as ornamental objects, or biotic elements at levels of their immediate ecosystem. This familiarity provides an opportunity to, not only introduce the concept, but also extend the exploration into a collaborative inquiry-based investigation. Last summer we presented a mini-workshop at the ABLE Conference at UNC (2012). Here we will comment on detailed steps of our protocol as well as summarize responses of workshop participants.

In order to focus participant attention and introduce our concept, a simple seed extract preparation is used as a model. The purpose is to measure protein expression using electrophoresis and compare the banding patterns among members of various plant families. In the past, this exercise was used successfully with students at different levels (high school and college) to introduce them to electrophoresis. At the ABLE workshop there was a consensus of teachers who felt that this procedure could be readily implemented at their respective campuses. Thus, we are suggesting a laboratory exercise wherein students will study the biochemistry of seeds using familiar vegetables to introduce a novel pedagogical approach to systematics. Hopefully students will intuit relationships among selected plants. Depending on their breadth of background, student teams can be encouraged to use these lab skills to explore whether such kinship holds for other members of various plant families.

Classification of plants has its roots in traditional methods of taxonomy. Floral arrangements (pistils and stamens) have provided the major bases. In this exercise there will be an investigation about whether these traditional features of classification are corroborated by analysis of biochemical characteristics. Seed protein expression will be assessed by examining protein banding patterns using PAGE (polyacrylamide gel electrophoresis). Based on our preliminary studies, two major plant families (*Brassicaceae* and *Fabaceae*) deserve attention. Most student have direct familiarity with these families through a balanced diet of cruciferous (e.g. broccoli, kale) and leguminous (e.g. string beans, peas) vegetables. Students can readily be challenged to think about the relatedness of members of these families, based first on their physical characteristics and then on their biochemical similarities. The lab encompasses learning tools at varying degrees of difficulty. The extent to which the instructor wishes to use them will depend on time constraints, status of student abilities, and available lab equipment. The dimension of inquiry-based collaborative learning with student teams will help to engage students on different levels of learning using Bloom's taxonomy.

Student Outline

Methodology - The Lab Protocol

I. Introduction to phylogenetics and botanical dietetics

A lecture on traditional methods of classification should first be presented by the instructor. It is useful to introduce students to basic organization of the plant using botanical flower and fruit models. A selected variety of leaves, fruits, and vegetables from the grocery store might be used to enhance the lesson. Alternatively, if time allows, students may germinate seeds and grow them to seedlings and mature plants. Fast-growing *Arabidopsis* (mustard) seeds complete their life cycle in one month. The plants can be examined to verify the features that underlie their classification. In addition, there are web sites that provide information about plant taxonomy. One source suggests the classification of the *Brassicaceae* (crucifer family): **http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=10120**.

To create interest the instructor might provide supermarket representatives of two families (for example, *Brassicaceae* and *Fabaceae*). These can be exhibited on the lab bench in random arrangements. Each team of students is asked to organize them according to some level of classification system of their own making. If there is more than one criterion used by students, they might be asked to explain how these criteria were used to classify these plants. Some students might feel that there was not enough information to make a judgment about classification. They should be ready to justify their position.

II. Protein analysis of seeds

Sample preparation

Seeds from different plant families are commercially available. After rinsing and drying, seeds are weighed (0.5g) and ground with mortar and pestle (Fig. 1). The mash is mixed with 3-5 ml distilled water for 2 minutes over ice. Since seeds are biochemically diverse, the volume of water necessary will depend on the presence of a visible slurry. The slurry is briefly vortexed and centrifuged at 10,000 rpm (4˚C, 2 X 10 min). Supernatant aliquots are delivered into separate microfuge tubes by decanting. This point can be considered a "stop point" and the samples may be frozen for later analysis.

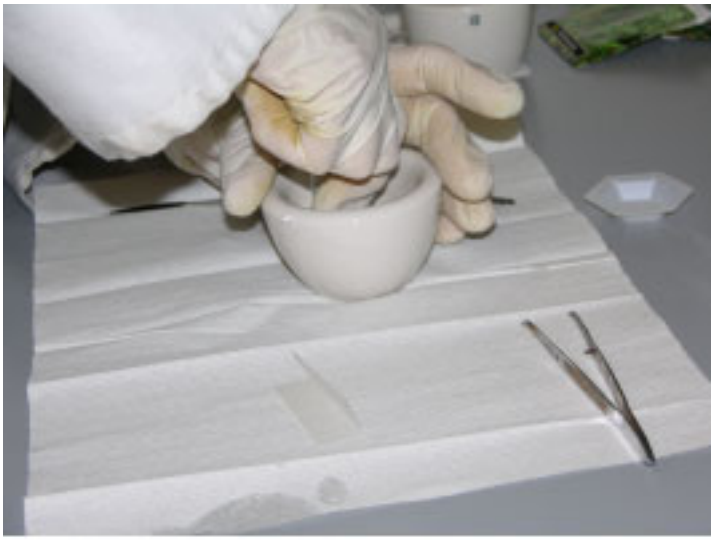

Figure 1. Seed preparation. After weighing, seeds are ground up with a mortar and pestle.

Protein Calibration

Total protein of each sample should be determined to equilibrate samples before loading each of the wells of the PAGE gel. We use the microassay procedure from *Bio-Rad* (**http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9004.pdf**) A calibration curve is first developed using different concentrations (0-10 µg /ml) of a known protein (BSA-bovine serum albumin). A spectrophotometer (595nm) measures the optical densities (OD) of known and unknown extracts (from 5µl of the seed supernatant) and these are compared along the curve to determine the protein concentration. Reliable OD readings are usually found along the lower range of concentrations of BSA (2-6 µg /ml) calibration curve (Fig. 2). The use of 1 ml cuvettes minimizes the quantity of extract. Using the template form of *Excel*™ is recommended in plotting the standard curve and calculating the volume of seed extract that will be needed to deliver 10 µg equivalent of protein to the wells on the electrophoretic gel during loading (described later).

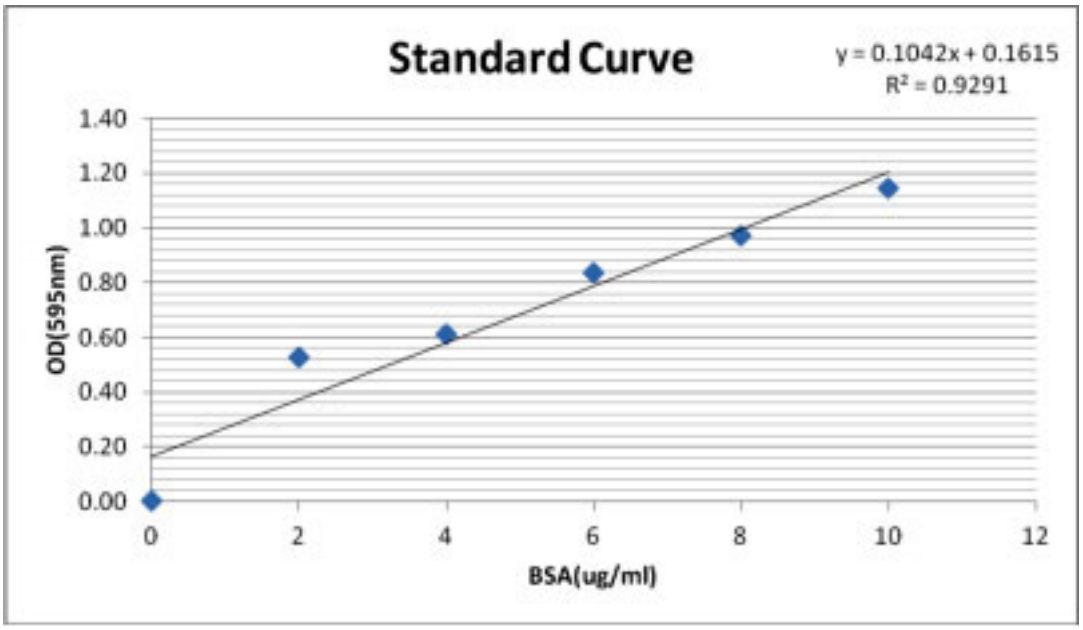

Figure 2. Protein calibration curve standardized against bovine serum albumen

Electrophoresis

This part of the exercise may require students to learn a new technique. Students may have been exposed to PAGE in another course. Depending on the proportions of students in each cohort, we suggest different approaches: (1) with most of the class familiar, spend little time demonstrating and begin the work on seeds; (2) with a 50/50 distribution, team the "knowers" with the "learners" and do a seed practice session; (3) with most of the class "learners," begin with a teacher demonstration.The technique of electrophoresis is generally known to most biologists although the protocols will differ slightly from one lab to another. An excellent simulation from Rochester Institute of Technology is available to help students understand the general principles (**http://people.rit.edu/pac8612/electro/Electro_Sim.html**). Two PAGE protocols can also be found in papers from previous ABLE Conferences (Racusen and Thompson, 1996; Frame, 2000).

Prior to electrophoresis, the stored frozen samples in labeled microfuge tubes are thawed in tap water and mixed under icy conditions with a thin glass pestle (engineered from melting the end of a Pasteur pipette). The samples are vortexed and centrifuged for five minutes in a *Serofuge* centrifuge (3400 rpm). The supernatant from the tissue extract (approximately 25 µl, depending on the protein content) is then diluted 1:1 with a sample buffer of "blue juice" (mixture of 950 µl Laemmli buffer and 50 µl mercaptoethanol). This new mixture is re-vortexed and re-centrifuged briefly. The mixture is subsequently placed in a 95 ˚C hot bath for five minutes. If a stop point is necessary, these samples can be stored as frozen extracts for future use.
When ready to perform PAGE, precast Tris-HCl gels (15% or 4-15% polyacrylamide gels, 10 wells with 50µl well capacity) were purchased. These are easily inserted into a vertical gel holder, after removing the comb and the adhesive strip. Tris-glycine-SDS buffer (available as a 10 X concentrate) is poured into the upper gel holder, after which the wells are loaded. The first and last lanes should contain a protein ladder with prestained color markers (8-10 proteins, 5 to 250 kD). The remaining wells are loaded with various unknown prepared extracts. Each well can be loaded with 10 µg of protein (in a volume of 20-25 µl). The volume of extract will depend on dilutions made in extract preparation and the total protein in your sample before dilution with the sample buffers. See protein calibration step detailed earlier.

Once loaded, the gel holder is placed in the electrophoresis chamber. The chamber is filled with Tris-glycine-SDS lower buffer. The banana plug electrodes of the gel holder are connected to the leads of a power supply, which is run at 150 V for approximately 45 minutes. The course of the gel run can also be monitored by observing the tracking dye. After the run, the gel is removed and notched at one end. The new TGX gels (sold by *Bio-Rad*) require use of a prying tool. After three washes with distilled water, the gel is stained in commercially available Coomassie Blue Bio-safe stain for 60 minutes; this stain can be re-used several times. Destaining with distilled water over the next 2-3 days is absolutely essential for clarifying the bands.

Data Analysis

The *NucleoVision* imaging system that was used is no longer commercially available; but other newer systems work as well. After scanning and preprocessing the image, the position and intensity of the bands in each of the lanes can easily be determined using the software which is used by the scanner. The intensity of the bands can be displayed as a graph over the length

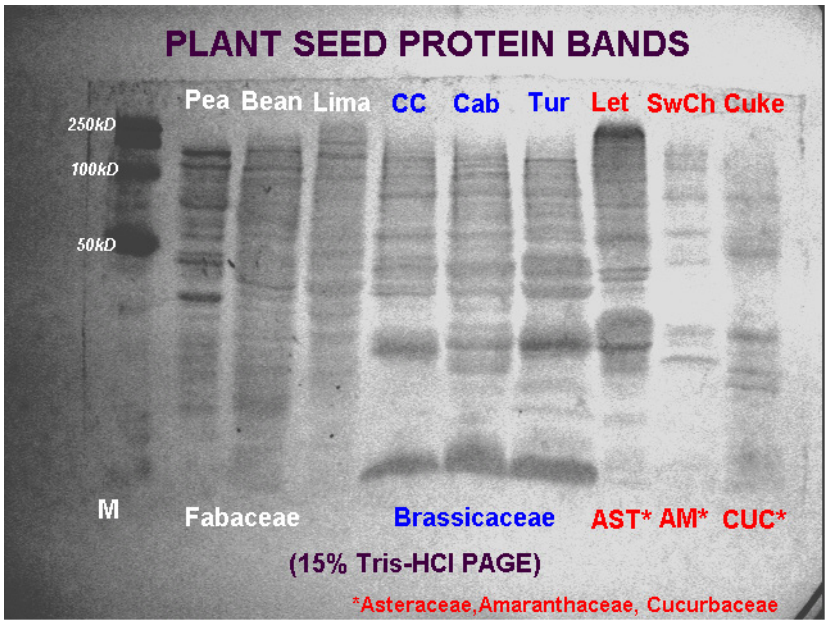

Figure 3. Electrophoresis of plant seed proteins showing the similarity in protein banding among members of the *Brassicaceae* (CC=Chinese cabbage, Cab=cabbage, Tur=turnip) and dissimilarity among members of *Fabaceae* (Pea=snow pea, Bean=bush bean, Lima=Lima bean). Also represented are Let=lettuce, SW=Swiss chard and Cuke=cucumber

of the lane. A comparison of the position and intensity of the protein bands for the various plant extracts is critical for analysis. After marking the bands, it is possible to determine whether there is coincidence between bands of different plant extracts. A similarity index (SI) can be employed (Vaughan and Denford, 1968). Similarity index = the number of coincidental band sets between two extracts/number of coincidental band sets + number of other bands counted in both comparison lanes. The molecular weight ladder of known protein standards allows students to estimate band molecular weights of the various protein bands. Pictures can then be annotated with titles and lane descriptions before exporting them as .jpg format and pasting them into a laboratory report.

An alternative approach to analysis would be to use a digital camera to photograph the gels after a run and save the images as .jpg or .tif files. One should preprocess the gel image using photo-editor software (from a photograph as a .tif or .jpg file or some scan) in order to increase contrast among bands. Pictures can then be annotated with titles and lane descriptions. A direct comparison of bands using Rf values (ratio of distance traveled by the unknown over the distance traveled by the front) is also a useful analytical tool.

Results

Based solely on appearance and physical characteristics of mature plants, students can be expected to group the members of the legumes in the same family (*Fabaceae*); the rationale for this is based on presence of pods. In contrast, because of their morphological diversity, samples from the *Brassicaceae* may not be identified as related. However, after surveying the protein bands of various members of this family, students might reconsider their initial classification. The seed protein patterns of *Fabaceae* are quite different but the banding patterns of *Brassicaceae* are very similar (Fig. 3).

The protein banding (fingerprint) pattern of members of the *Brassicaceae* shows clear similarities, despite the obvious morphological differences among adult plants. Using similarity index (SI) computation, there are clear trends which suggest close relationships among cruciferous vegetables (Table 1). Indices greater than 0.4 suggest similarity of proteins between families. The students who do the analysis and literature search will realize that the members of this family are related by genus as well as by species. Cabbage, cauliflower, broccoli, kale, kohlrabi, and Brussel sprouts all belong to the same genus and species: *Brassica oleraceae*. Their chromosome numbers are also the same (2n=18). Yet, there are marked differences in the morphology of the mature plant featuring differential development of the bud, leaf, or stem.

In contrast, there is less similarity among the protein banding of seeds of bush bean, Lima bean and sugar-snap pea. Much of this may be a function of the uniqueness of each genus and species. Lima bean, garden bush bean and sugar snap pea are more distantly related (*Phaseolus lunatus*, *Phaseolus vulgaris*, and *Pisum sativum* respectively). Their chromosome numbers are also somewhat different; P. sativum has 14 compared with 22 in the others.

Table 1. Similarity indices between seed* protein profiles.

	Pea	Bean	Lima	ChCab	Cabbage	Turnip	SwChard	Lettuce	Cucum
Pea		0.29	0.23	0.25	0.20	0.26	0.19	0.23	0.11
Bean	0.29		0.24	0.10	0.16	0.26	0.20	0.25	0.12
Lima	0.23	0.24		0.12	0.04	0.18	0.15	0.24	0.14
ChCab	0.25	0.10	0.12		0.63	0.44	0.29	0.24	0.16
Cab	0.20	0.16	0.04	0.63		0.50	0.31	0.19	0.11
Turnip	0.26	0.26	0.11	0.44	0.50		0.36	0.18	0.31
SwChard	0.19	0.20	0.15	0.29	0.31	0.36		0.17	0.06
Lettuce	0.23	0.25	0.24	0.24	0.19	0.18	0.17		0.04
Cucum	0.11	0.12	0.14	0.16	0.11	0.31	0.06	0.04	

(*see Fig. 3 for legend)

Notes for the Instructor

There remains a paucity of information about the use of molecular taxonomy in botanical classification. Plants are known for their aneuploidy. A number of families (e.g. *Brassicaceae* and *Poaceae*) are also characterized by polyploidy. Such plants display overt changes in morphology which, in the case of the *Poaceae* (grains), has resulted in an increase in its nutritive yield. In contrast, in vertebrates, such chromosomal variation cannot occur without serious abnormalities and / or morbidity. Even less marked changes in plant ploidy can result in subspecies which are easily mistaken for unrelated specimens. This is clearly seen in the *Brassicaceae*, where different cultivars of *Brassica olecaceae* have produced plant parts that are differentially developed. Compare the leafy cabbage, to the floral cauliflower, to the tough stems of broccoli!

These relationships suggest that the ontogeny of plants may be far more complex and quite different from animals. Although protein banding patterns are similar in the seeds of members of *Brassicaceae*, there can be subtle differences in the protein composition of these bands which suggest variations in the number and kind of transcriptional and growth factors in different members of this family. Could this explain the differences in morphological features? Consider the possibility of alterations in rDNA synthesis between 2 members of the *Fabaceae* (Almeida and Pedrosa-Harand, 2011). There may also be subtle differences in protein composition within bands due to unique growth factors and storage proteins. Although this exercise does not, in itself, address these issues, such questions might arise in class discussions after the students have finished the exercise.

This exercise was never designed as a definitive work on plant development and systematics. It is instead an opportunity for students to collaborate and show interest in a biological phenomenon. It is a springboard for further studies. For example, after the course is done and the reports are collected, perhaps one of the students might continue by exploring other members of the family of *Brassicaceae* (over 3000 species). In our summer workshop, the consensus among our 23 participants was that this exercise was "novel, clear, organized, simple, neat and cool". They enjoyed the hands-on parts of the experimental lab. The majority of these teachers expressed the opinion that this approach has the potential to engage students, teach them some basics of molecular techniques and sensitize them to the complexities of plant taxonomy.

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