Post-Lab Activity

Analysis and Interpretation of Results

Detailed Gel Analysis

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 below, 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.

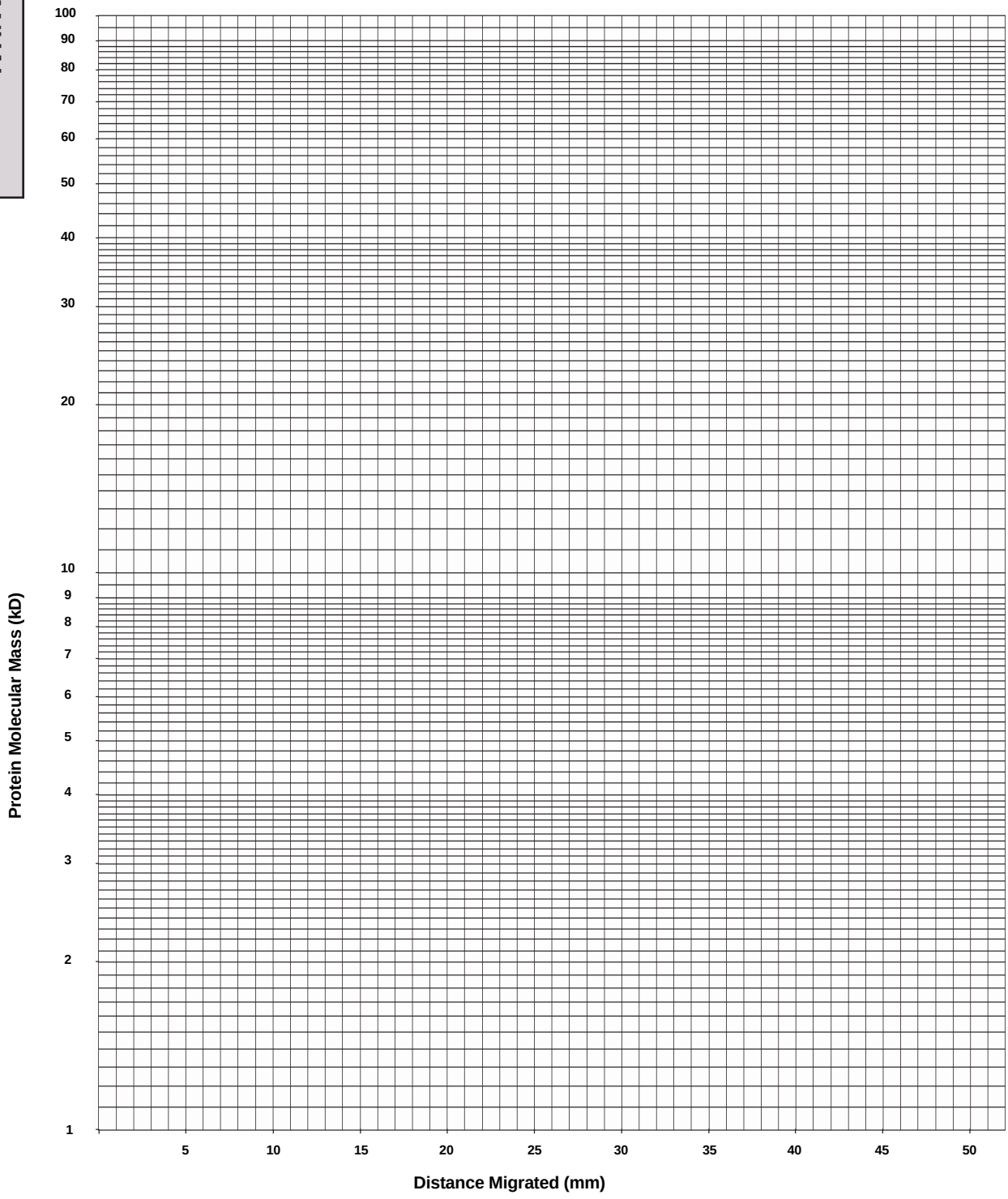


Fig. 16. Two-cycle semi-logarithmic graph paper to construct curve of the protein molecular mass against the distance migrated.

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 in 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,

Determine which fish have each characteristic (protein)

Make a table with a row for every band size you have recorded and a 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 below).

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**

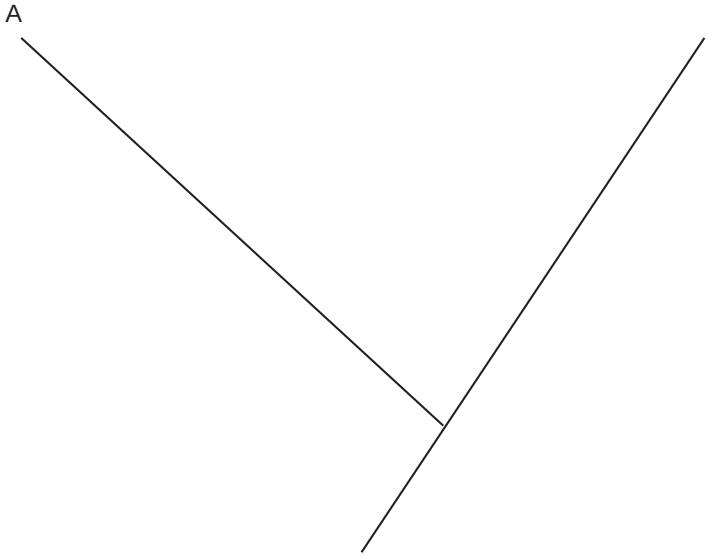
Find the number of characteristics shared by each of the fish

In the table below both the row and column headings are the types of fish. From the table above, 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 2 bands in common while species B and C have 10 bands in common. The table below will be the basis for drawing your cladogram.

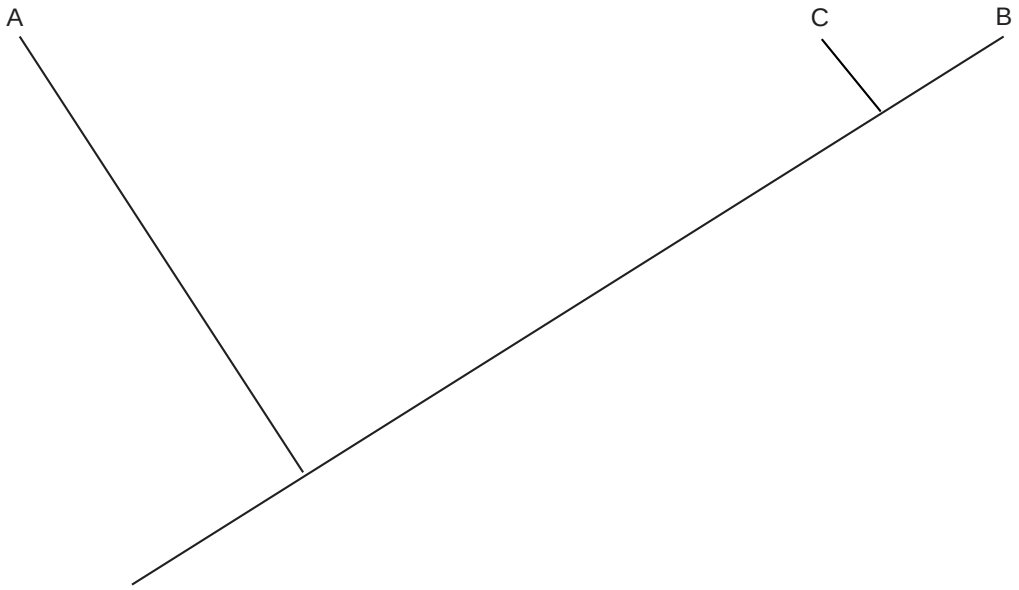
	Species A	**Species B**	**Species C**	**Species D**	**Species E**
Species A	8	2	2	2	2
Species B		10	10	5	3
Species C			13	5	4
Species D				10	2
Species E					12

Construct your cladogram

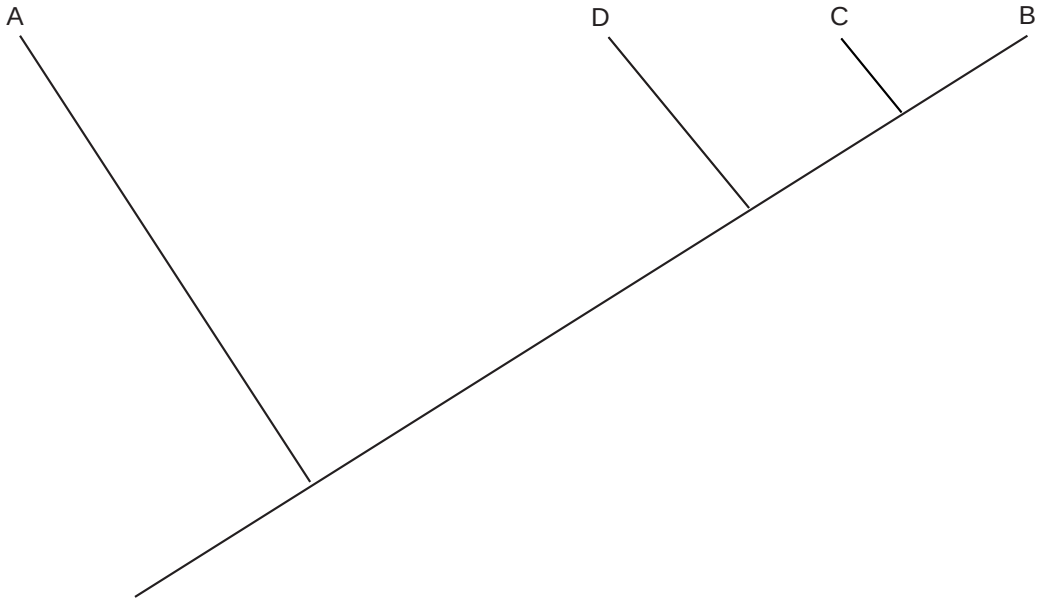
Now you are ready to construct your cladogram. First draw a line to form the trunk of your cladogram. Find the fish with the least bands in common. In the example above it is species A, which has only 2 bands in common with any of the other fish. Then draw a side branch off the line near the bottom of 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.



Now, 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).



Now, 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.

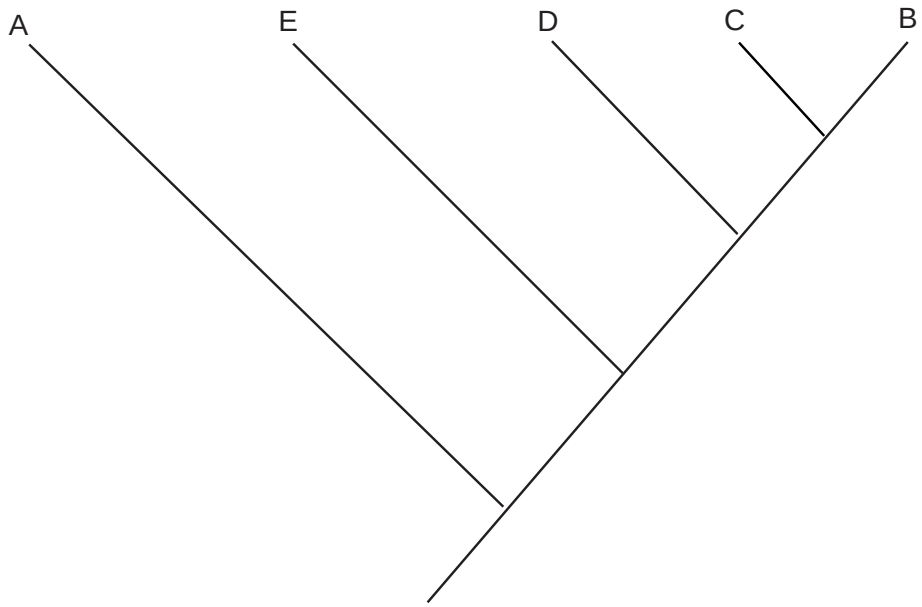


The last fish to add to the cladogram in this example is species E, which shares four bands with species C, three bands with species B, and only two bands with species A and D. This fish may seem trickier to place than the others because it shares more characteristics with species B and C than it does 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.



Now compare your cladogram with your original predictions. Write your deductions below.

Appendix A: Protein Electrophoresis Using Agarose Gels and Horizontal Gel Electrophoresis Apparatus

The principles of protein separation by electrophoresis can be taught using agarose gels and horizontal electrophoresis apparatus that is typically used for separating DNA. Proteins are rarely separated on agarose in the real world because proteins are much smaller than DNA (compare the number of carbons in a base pair to an amino acid). Polyacrylamide has a tighter matrix than agarose which, in addition to the discontinuous system used in SDS-PAGE (see Lesson 1: Introduction to Protein Electrophoresis SDS-PAGE), is much better at resolving most proteins into distinct bands. However, agarose gels are used in scientific laboratories to separate large proteins, for example serum proteins from blood. The following protocol describes a method to separate fish muscle proteins using agarose gels and horizontal gel electrophoresis apparatus. The same experimental principles apply to this method as SDS-PAGE, such as the SDS conveying a net negative charge and the denaturation of proteins using heat. The main difference is that the gel is not a discontinuous system like the polyacrylamide gels.

An example of the results is shown in Figure 18 below. You can see that the bands are not as distinct as with polyacrylamide gels, and that the larger proteins separate better with more distinct bands than the smaller ones, which diffuse in the more porous agarose.

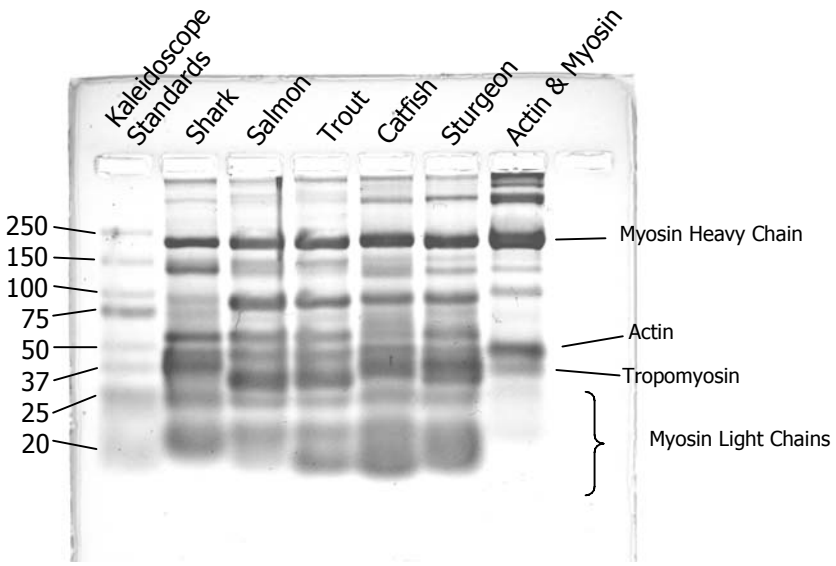


Fig. 18. 4% agarose gel electrophoresed at 100 V for 45 minutes, fixed, stained with Bio-Safe Coomassie stain, and destained in water.

It is important to note that both the type of agarose and the running buffer are different from what is usually used to separate DNA. **Regular agarose will not work with this protocol**. The agarose used to make these gels is high-percentage PCR low-melt agarose and must be purchased separately from the kit (Certified PCR low-melt agarose, 25 g, catalog #161-3113). This special type of agarose is superior to regular molecular biology agarose at separating very small molecules. Low-melt agarose is different from regular agarose because once dissolved and formed into gels, it melts at around 65°C and will remain molten to around 34°C. Therefore, if the gels are prepared in a hot climate, it may be necessary to put the gels in the refrigerator to set. **Tip**: Thinner agarose gels (e.g., 5 mm) will resolve proteins better than thick gels, so pour your gels carefully with minimal agarose. The running buffer and the buffer